A RATIONALE FOR AN ANALYSIS OF RNA REPLICATION*

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The intent is to trace our efforts at understanding the molecular life history of RNA genomes using the E. coli–RNA phage system. In the process, an attempt will be made to provide a background of the thinking that went into the experiments to be described. Because of the usual dominance of serendipity in biological and biochemical research, it is not often that it is either informative or useful to record the reasons for a particular set of experiments; noting the outcome is usually sufficient. However, the present instance may represent an exception.

Problem of Communication between an RNA Virus and Its Host Cell.—We start with the fact that all organisms which use RNA as genetic material are mandatory intracellular parasites. They must, therefore, carry out a major part of their life cycle in cells which use DNA as genetic material and RNA as genetic messages. On entry, the viral RNA is faced with the problem of inserting itself into the cellular information flow pattern in order to communicate its own instructions to the synthesizing machinery. A possibility one might entertain centers on whether an RNA virus employs the DNA-to-RNA pathway of information flow. This could occur either because the DNA of the host already contains a sequence homologous to the viral RNA (i.e., the “escaped genetic message” hypothesis), or because such DNA sequences are generated subsequent to infection by reversal of the DNA-dependent RNA synthesizing reaction. It is clear that a decision on the existence or nonexistence of homology between viral RNA and the host DNA is a necessary prelude to further experiments designed to delineate the molecular life history of an RNA genome.

To answer questions of this nature, Doi and Spiegelman employed the specific hybridization test combined with the subsequently developed use of RNase to eliminate “noise.” The sensitivity required had already been achieved in earlier experiments which identified the DNA complements of sRNA and ribosomal RNA. Under conditions where complexes between 23S rRNA and DNA were readily observed, none were detected between the viral RNA and the infected host DNA.

The negative outcome of the hybridization test implies that the DNA-to-RNA pathway is not employed, from which it follows that these RNA viruses must have evolved a mechanism of generating RNA copies from RNA. The existence is then predicted of an enzymatic mechanism involving an RNA-dependent RNA polymerase which we have named “replicase” for purposes of brevity and alliterative usefulness.

It seems highly unlikely that an enzyme of this sort pre-exists in the cell. All recognized cellular RNA components, including the message fraction, the two ribosomal components, and the translational 4S RNA, have been shown to be complementary to some sequences in the homologous DNA. Furthermore, actinomycin D which inhibits the DNA-dependent RNA polymerase (transcriptase) pre-
vents synthesis of RNA in both bacterial\(^\text{12}\) and animal cells, but does not inhibit the production of RNA viruses.\(^\text{13}\)

(2) **Viral RNA as a Translatable Message.**—The arguments above led us to assume that no mechanism pre-existed in uninfected cells for generating either complements or identical replicas from RNA. It was our feeling from the outset that the transcriptase reaction would not normally be employed as a step in virus replication since its ability to employ RNA templates is poor and fragmentary. Consequently, when we began our enzymological investigations, much of our effort was directed at eliminating transcriptase from our preparations. Complete justification of this view was ultimately provided \([\S 5 \text{ and } 6]\) when purified replicases were obtained and examined. In any event, this line of reasoning did lead to the prediction that the entering RNA must itself serve as a protein program and be directly translatable in order to communicate with the cell. Direct proof that viral RNA is directly translatable into protein was achieved in an \textit{in vitro} system by Nathans \textit{et al.}\(^\text{14}\) with \textit{f2} and confirmed by Ohtaka and Spiegelman\(^\text{15}\) with MS-2 and by Clark \textit{et al.}\(^\text{16}\) with STNV.

There is another consequence of this line of reasoning which can be, and was, subjected to test. Since the new kind of replicase must be synthesized \textit{before replication can begin}, it follows that the entering RNA must be conserved while serving as a protein program. Without conservation, there would be nothing left to replicate by the time the replicase was completed. Doi and Spiegelman\(^\text{17}\) undertook to test the validity of this prediction by the use of MS-2 in which the RNA was doubly labeled with N\(^\text{15}\) and P\(^\text{32}\). Both isotopes were recovered in the same strand, leading to the conclusion that the parental strand of an RNA virus is completely conserved during all the replications and translations required to produce a full yield of mature virus particles.

(3) **Possible a priori Mechanism of RNA Replication.**—Before we attempt to detail the more recent enzymological approaches to the problem of RNA replication, it is of interest to review briefly the various possibilities that can be entertained about the nature of RNA replication as derived from arguments of varying plausibility. The kinds of readily imaginable mechanisms can be divided into two classes: (1) Nature is pleasantly uniform, the nucleic acid universe being completely describable by \textit{“Watson-Crickery.”} Consequently, the replication of single-stranded RNA will mimic the mechanism employed by its counterpart, single-stranded DNA. (2) The alternative view considers the possibility that RNA genomes evolved a different duplicating device. We may now consider the \textit{a priori} reasons which can be marshaled in favor of each line of thought.

(a) **A variant solution to RNA replication:** In the first place, one can plausibly argue that since RNA genomes are found \textit{only} in mandatory intracellular parasites, they must have arisen after cells evolved. Consequently, the RNA viruses emerged in a complicated biochemical environment containing highly complex enzyme molecules fashioned to carry out reactions demanding refined levels of selective specificity. These were available to the RNA viruses for choice and modification to suit their particular needs. In contrast, DNA genomes emerged in the primitive biochemical environment which characterized \textit{“genesis,”} in which the problem of replication had to be solved unaided by the subsequently developed sophisticated protein catalysts. Of necessity, DNA was initially forced to use its
hydrogen-bonding capacities and employ the principle of complementarity to duplicate. The apparent use of this mechanism today may represent a residue of the difficulties which DNA encountered in its early evolution. We emphasize the qualification “apparent” since, despite a widespread belief to the contrary, the fact is that we do not know how DNA duplicates in detail. No one has, as yet, produced incontrovertible evidence which compels acceptance of base pairing on the template as a step which necessarily precedes the addition of the next complementary residue to the growing chain. One can still entertain a mechanism in which the enzyme makes the choice via “allosteric instruction” from the template; base pairing could then occur subsequent to the synthesis of the new diester bond.

Another argument one can offer in favor of a unique solution for RNA replication stems from the fact that RNA genomes are translatable messages. In view of what we know about the coding dictionary, the complement of a translatable message is likely to be nonsense. Complementary transcription of the original strand is therefore not only unnecessary for information transmission to the cell, but is in fact useless. Consequently, if complementary copying does occur as an intermediate step, it would be employed only for replicative purposes. Synthesis of polynucleotide strands is energetically very expensive, and avoiding this step would provide an obvious advantage to RNA viruses. In any event, because of these and other considerations, we pointed out that RNA viruses could furnish us with a surprising variation on the Watson-Crick theme.

(b) DNA-like solutions for RNA replication: The most popular and widely adopted model stems from the studies of the single-stranded DNA virus \( \Phi X-174 \), the relevant properties of which may be briefly summarized. On infection, the single-stranded DNA is converted into a double-stranded structure which has been named the “replicative” form (RF-DNA). It can be shown by column chromatography and prelabeled virus that this conversion is complete even at elevated multiplicities of infection.

It is presumed that the RF-DNA then serves as a template for the formation of single-stranded copies via an asymmetric synthesis, analogous to transcription into RNA. However, proof that the RF-DNA is a “replicative” form remains to be provided. Hayashi and Spiegelman showed that the single strand found in the mature virus particle is the nonsense strand. It follows that in order for this virus to communicate with its host, the complement must be synthesized. As a consequence, finding a double-stranded structure does not necessarily signify that the “replicative” form has been identified; its presence is already justified by its requirement for transcription. Indeed, even if a single-stranded DNA virus were discovered carrying the coding strand, we would still predict the intervention of a duplex. The cellular transcription mechanism, which DNA viruses must use, is designed to make single-stranded copies of RNA from a double-stranded template so that, in any event, the duplex would have to be completed.

Despite these reservations, one must grant the attractiveness of assuming that a single-stranded RNA virus would have the same general problems as a single-stranded DNA and that therefore RNA might well adopt the same pathway for its life cycle. Thus, Ochoa et al. proposed that the first step in the replication of an RNA would be the conversion of the incoming RNA into a double-stranded structure which could then serve as a “replicative” form for the generation of single-
stranded copies of the mature viral RNA. According to this view, replication of RNA viruses introduces no novelties. The same general rules, assumed to function in the φX-174 system, are presumed to apply here also.

(4) The Search for a Double-Stranded RNA.—Not only was the idea of a duplex replicating form attractive, but what appeared to be supporting evidence quickly accumulated in the literature on the RNA bacteriophages. The following properties can be used in a search for evidence of double-stranded RNA: (1) comparative resistance to RNase, (2) a lower density in a Cs₂SO₄ gradient, and (3) conversion to RNase sensitivity by heating and fast cooling at low ionic strength. Structures possessing one or more of these properties were found in infected cells. We have elsewhere summarized similar findings in our own laboratory.

Everything looks reasonable and consistent with the interpretation involving a double-stranded intermediate in RNA replication. However, closer examination reveals certain difficulties in accepting this as compelling evidence that a component in the replicative pathway of viral RNA has been unveiled. The following disturbing properties of the resistant structure may be noted: (1) Only a very small percentage of the injected viral RNA strands is to be found in this structure; and (2) the resistant material tends to accumulate late in infection, long after many mature single strands have been made, a feature not easily reconcilable with the resistant structure (RS) being a mandatory initial intermediate. These findings are in striking contrast to the φX-174 situation, where all injected strands are converted into RF-DNA and where RF-DNA appears in the first stages of infection, long before appearance of single-stranded viral DNA.

One can argue that since resistant structures are found only in infected cells, they must have some relevance to replication. While plausible, such arguments do not have the ring of logical necessity. One can grant that the resistant structure is a consequence of the infective process without accepting it as an intermediate of the replicative process. It may play some other undetermined role or be a nonfunctional artifact. One must bear in mind that we are dealing here with infected cells which are well along the path to death, and it is not outside the realm of possibility that pathological artifacts might be produced. Under these circumstances, more direct evidence than mere existence must be provided before the resistant structures are accepted as demonstrated components of the replicative mechanism.

In any case, our own attempts and those of others to study the process in the infected cell convinced us that it would be difficult to design a truly decisive experiment which could hope to settle the question of the relation of the RNase-resistant structures to RNA replication. It seemed necessary to get on with the enzymology in the hope that the relevant enzyme system could be purified to the point where the mechanism of RNA synthesis could be examined in a simple system permitting hard inferences.

(5) The Search for the MS-2 Replicase.—The search for a unique RNA-dependent polymerase is complicated by the presence of a variety of enzymes which can incorporate ribonucleotides either terminally or subterminally into pre-existent RNA chains. In addition to transcriptase, which can use RNA templates, there are other enzymes (e.g., RNA phosphorylase, polyadenylate synthetase, etc.) which can mediate extensive synthesis of polyribonucleotide chains. It is obvious that a claim for a new type of RNA polymerase must be accompanied by
evidence for RNA dependence and a demonstration that the enzyme possesses some unique characteristic which differentiates it in one or more of its properties from previously known enzymes with which it can be confused.

In addition to these enzymological difficulties, we recognized a biological feature of the situation which influenced, in at least one important detail, the procedure we chose in the search for replicase. The point at issue may perhaps best be described in rather naive and admittedly somewhat anthropomorphic terms. Consider an RNA virus approaching a cell some $10^6$ times its size and into which the virus is going to inject its only strand of genetic information. Even if the protein-coated ribosomal RNA molecules are ignored, the cell cytoplasm still contains approximately 10,000 free RNA molecules of various sorts. If the new replicase were indifferent and replicated any RNA it happened to meet, what chance would the single original strand injected have of multiplying?

Admittedly, there are several ways out of this dilemma. One could, for example, isolate the new polymerase molecule and the viral RNA in some sequestered corner where they would be undisturbed by the mass of cellular RNA components. However, we entertained the unique possibility that the virus is ingenious enough to design a polymerase which would recognize its own genome and ignore all other RNA molecules.

At the outset, of course, we did not know which solution had been adopted by the virus to solve this dilemma, or even if the dilemma was real. However, the possibility that it did exist, and that replicase selectivity might be the chosen solution, required that its implications not be ignored for, if true, its disregard would guarantee failure. In particular, this view meant that we could not afford the luxury of employing any conveniently available RNA in the search for replicase. It demanded the use of purified viral RNA in all steps of the purification. Further, one might perhaps push the selectivity property to its ultimate pessimistic conclusion. If the cleverness of the replicase extends further, it might well be true that even a fragment of its own genome would not be recognized and accepted for replication. This added possibility made it necessary to provide a guarantee that the RNA employed is not only homologous, but also intact. This in turn introduced the complication that stages of purification preceding the removal of ribonuclease could well yield ambiguous or indeed false clues even with intact homologous RNA. Thus, one had to "fly blind" initially and depend on very brief assays to provide the guides for the direction of the subsequent steps.

Despite all these potential obstacles, most of which were actually realized, the first success was achieved in 1963. A procedure involving negative protamine fractionation and column chromatography yielded what looked like the relevant enzyme from *E. coli* infected with MS-2. Most important of all, the preparation exhibited a virtually complete dependence on added RNA, permitting a test of the expectation of specific template requirement. The response of MS-2 replicase to various kinds of nucleic acid revealed a striking preference for its own RNA. No significant activity was observed with either the host sRNA or ribosomal RNA. The ability of the replicase to discriminate between one RNA molecule and another does indeed solve the crucial problem for an RNA virus attempting to direct its own duplication in an environment replete with other RNA molecules. By producing a polymerase which ignores the mass of pre-existent cellular RNA, a guarantee is provided that
replication is focused on the single strand of incoming viral RNA, the ultimate origin of progeny.

It seems worth noting that sequence recognition by the enzyme can be of value not only to the virus, but also to the investigator. As already noted, the search for viral RNA replicases must perforce be carried out in the midst of a variety of highly active cellular polymerases capable of synthesizing polynucleotides. If the enzyme finally isolated possesses the appropriate template requirement, a comforting assurance is furnished that the effort expended and the information obtained are indeed relevant to an understanding of RNA replication.

(6) Confirmation of Specific Template Requirements of RNA Replicases.—Our line of reasoning would lead to the expectation that RNA replicases induced by other RNA viruses would show a similar preference for their homologous templates. However, this was not a foregone conclusion since it was conceivable that other viruses might evolve different solutions to the problem of preferential synthesis. It seemed important to determine whether template selectivity could be observed in another virus unrelated to MS-2. The Q9 phage of Watanabe was chosen because of its serological and other chemical differences.

The isolation and purification of the Q9 replicase essentially followed, with slight modifications, the procedures worked out earlier for the MS-2 replicase. The properties of the Q9 replicase on purification to the stage of complete RNA dependence exhibited the same general features as had been observed with MS-2 replicase, including requirements for all four triphosphates, and Mg++. Figure 1 shows the kinetics observed in a reaction mixture containing saturating amounts of template (1 y of RNA for 40 y protein). Continued synthesis is observed at 35° for periods exceeding 5 hr, and in 2 hr the amount of RNA synthesized corresponds to five times the input of template. By variation in the amount of RNA added and the time permitted for synthesis, virtually any desired level of increase over the starting material can be achieved. The cessation of synthesis within 5-10 min reported by others for presumably similar preparations has been observed by us only in the early stages of purification prior to the removal of the nucleases.

The abilities of various RNA molecules to stimulate the Q9 replicase to synthetic activity at saturation concentrations of homologous RNA are recorded in Table 1. The response of the Q9 replicase is in accord with that reported for the MS-2

![Figure 1: Kinetics of replicase activity](image-url)
TABLE 1

RESPONSE OF Q8 REPLICASE TO DIFFERENT TEMPLATES*

<table>
<thead>
<tr>
<th>Template</th>
<th>Incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q8</td>
<td>4,929</td>
</tr>
<tr>
<td>TYMV</td>
<td>146</td>
</tr>
<tr>
<td>MS-2</td>
<td>35</td>
</tr>
<tr>
<td>Ribosomal RNA</td>
<td>45</td>
</tr>
<tr>
<td>tRNA</td>
<td>15</td>
</tr>
<tr>
<td>Bulk RNA from infected cells</td>
<td>146</td>
</tr>
<tr>
<td>Satellite virus of tobacco necrosis</td>
<td>61</td>
</tr>
<tr>
<td>DNA (10 µg)</td>
<td>36</td>
</tr>
</tbody>
</table>

Conditions of assay are those specified in Fig. 1. RNA-dependent activity was assayed at 1 µg of RNA for each 40 µg of protein, and DNA-dependent activity at 10 µg of DNA. Control reactions containing no template yielded an average of 30 cpm.

* Haruna and Spiegelman (ref. 40).

replicase, the preference being clearly for its own template. The only heterologous RNA showing detectable activity is TYMV, and it supports a synthesis corresponding to 3 per cent of that observed with homologous Q8 RNA. Both of the heterologous viral RNA's (MS-2 and STNV) are completely inactive, and, again, so are the ribosomal and transfer RNA species of the host cell. It is important to note that, as in the case of MS-2 replicase, the absence of response to DNA shows that our purification procedure eliminates detectable evidence of transcriptase from our enzyme preparations.

The following features distinguish the purified replicases described here from the presumably similar preparations reported* by other laboratories: (1) complete dependence on added RNA, (2) competence for prolonged (more than 5 hr) synthesis of RNA, (3) ability to synthesize many times the input template, (4) saturation at low levels of RNA, and (5) virtually exclusive requirement for homologous template under optimal ionic conditions.

It should be evident from the properties listed that the replicases were indeed approaching a state of purity where it became relevant to examine the nature of the product in greater detail—a necessary prelude to experiments designed to illuminate mechanism.

The experimental analysis of a replicating reaction centers necessarily on the nature of the product. If, in particular, the concern is with the synthesis of a viral nucleic acid, data on base composition and nearest neighbors, while of interest, are hardly decisive. The ultimate issue is whether or not replicas are in fact being produced. To answer this question, information on the sequence of the synthesized RNA is required. Affirmative evidence of similarity between template and product would provide assurance that the reaction being studied is indeed relevant to an understanding of the replicative process.

(7) Autocatalytic Synthesis of a Viral RNA.—The ability of a replicase to distinguish one RNA sequence from another can be used to provide information pertinent to the question of similarity of product to the template. Two sorts of readily performed experiments can decide whether the product is recognized by the enzyme as a template. One approach is to examine the kinetics of RNA synthesis at template concentrations which start below those required to saturate the enzyme. If the product can serve as a template, a period of autocatalytic increase of RNA should be observed. Exponential kinetics should continue until the product saturates the enzyme, after which synthesis should become linear.
FIG. 2.—Kinetics of RNA synthesis. A 2.5-ml reaction mixture was set up containing the components at the concentrations specified in the legend for Fig. 1. The mixture contained 400 μg of enzyme protein and 2 μg of input Qβ-RNA so that the starting ratio of template to enzyme was one fifth of the saturating level. At the indicated times, 0.19-ml aliquots were removed and assayed for radioactive RNA as detailed in Fig. 1. The ordinates for cpm and μg of RNA synthesized refer to that found in 0.19-ml samples. The data are plotted against time arithmetically on the right, and semilogarithmically on the left. The arrows indicate change from autocatalytic to linear kinetics (Haruna and Spiegelman).

A second type of experiment is a direct test of the ability of the synthesized product to function as initiating template. Here, a synthesis of sufficient extent is carried out to ensure that the initial input of RNA becomes a quantitatively minor component of the final reaction mixture. The synthesized RNA can then be purified and examined for its template functioning capacities, a property readily examined by means of a saturation curve. If the response of the enzyme to variation and concentration of product is the same as that observed with the viral RNA, one would have to conclude that the product generated in the reaction is as effective a template for the replicase as is RNA from the mature virus particle.

Preliminary experiments established that 40 μg of enzyme protein was saturated by approximately 1 μg of Qβ-RNA. An experiment was therefore set up in which the ratio of input template to protein was 1/5 of the saturation value. The results are plotted in Figure 2 arithmetically and semilogarithmically to permit ready comparison of kinetics. Exponential increase of RNA is evident over a period of approximately 3 hr. The arrows indicate the time at which the kinetics depart from exponential and become linear. Extrapolation to the ordinate indicates that the change to linear synthesis occurs when approximately 1 μg of RNA has accumulated.

The results just described are consistent with the implication that the product produced in the course of the reaction can serve to stimulate new enzyme molecules to activity. The enzyme is therefore able to recognize the product as being one which is homologous to its own genome.

To carry out the more direct test of this conclusion, a 1-ml reaction mixture was
set up and the synthesis allowed to proceed for 3.5 hr, by which time a more than 60-fold increase of the input material was achieved. The reaction was then terminated and the RNA purified by the phenol method which yielded 55 per cent of the synthesized product. Examination in a sucrose gradient showed that much of the product has the 28S size characteristic of Q3 RNA.

Figure 3 illustrates the response of the replicase to various input levels of the product (triangles) compared to the original viral RNA (circles). It is evident that the RNA synthesized is as effective in serving as a template as the original viral RNA.

The data just summarized support the assertion that the reaction generates a polynucleotide of the same molecular weight ($1 \times 10^6$) as viral RNA and which the replicase cannot distinguish from its homologous genome. It is clear that the enzyme is faithfully copying the recognition sequence employed by the replicase to distinguish one RNA molecule from another.

(8) Synthesis of an Infectious Self-Replicating Viral RNA.—The next question concerns the extent of the similarity between product and template. Have identical replicas been in fact produced? The most decisive test would determine whether the product contains all the information required to program the synthesis of complete virus particles in a suitable test system. The success we have just recorded encouraged an attempt at this next phase of the investigation which would subject the synthesized RNA to this more rigorous challenge. In the experiments to be described, all RNA preparations were first phenol-treated prior to assay. Further, the phenol-purified synthetic RNA was routinely tested for whole virus particles by assay on intact cells, and none were found in the experiments reported.

We now summarize experiments in which the kinetics of the appearance of new RNA and infective units were examined in two different ways. The first shows that the accumulation of radioactive RNA is accompanied by a proportionate increase in infective units. The second proves by a serial dilution experiment that the newly synthesized RNA is infectious.

(a) Comparison of the kinetics of appearance of RNA and infectious units: To compare the appearance of newly synthesized RNA and the presence of infectious units in an extensive synthesis, a reaction mixture was set up containing the necessary components at the concentrations required. Aliquots were taken at the times indicated for the determination of radioactive RNA and purification of the product for infectivity assay. Figure 4 shows the observed increase in both RNA and
infectious units. The amount of RNA (0.8 γ per ml at 0 time) is well below the saturation level of the enzyme present. Consequently, the RNA increases autocatalytically for about the first 90 min, followed by a synthesis which is linear with time. We note that the increase in RNA is paralleled by a rise in the number of infectious units.

Experiments carried out with other enzyme preparations yielded results in complete accord with those just described. Another example is given in Figure 5 in which the enzyme used was purified pycnographically in a cesium chloride density gradient which decreases the virus particle content by a factor of 10^6 without change in the properties of the enzyme. An examination here reveals that again one has parallel increases in both RNA and infectious units.

(b) Proof that the newly synthesized RNA molecules are infectious: The kind of experiments just described offer plausible evidence for infectivity of the newly synthesized radioactive RNA. However, they are not conclusive since they do not eliminate the possibility that the agreement observed is fortuitous. One could argue, however implausibly, that the enzyme is “activating” the infectivity of the input RNA while synthesizing new noninfectious RNA, and that the rather complex combination of exponential and linear kinetics of the two processes coincides fortuitously.

Direct proof that the newly synthesized RNA is infectious can, in principle, be
obtained by experiments which employ N\textsuperscript{15}-H\textsuperscript{3}-labeled initial templates to generate N\textsuperscript{14}-P\textsuperscript{32} product. The two can then, in principle, be separated in equilibrium density gradients of cesium sulfate. Such experiments have been carried out for other purposes and will be described elsewhere. However, the steepness of the cesium sulfate gradient makes it difficult to achieve a separation clean enough to be completely satisfying.

There exists, however, another approach which bypasses these technical difficulties by taking advantage of the biology of the situation and of the fact that we are dealing with a presumed self-propagating entity. Consider a series of tubes each containing 0.25 ml of the standard reaction mixture, but no added template. The first tube is seeded with 0.2 \( \gamma \) of Q\textbeta\ RNA and incubated for a period adequate for the synthesis of several \( \gamma \) of radioactive RNA. An aliquot (50 \( \lambda \)) is then transferred to the second tube which is in turn permitted to synthesize about the same amount of RNA, a portion of which is again transferred to a third tube, and so on.

If each successive synthesis produces RNA which can serve to initiate the next one, the experiment can be continued indefinitely and, in particular, until the point is reached at which the initial RNA of tube 1 has been diluted to an insignificant level. In fact, enough transfers can be made to ensure that the last tube contains less than one strand of the input primer. If, in all the tubes, including the last one, the number of infectious units corresponds to the amount of radioactive RNA found, convincing evidence is offered that the newly synthesized RNA is infectious.

A complete account of such a serial transfer experiment may be found in Spiegel-

![Figure 6](image-url)
Aside from controls, 15 transfers were involved, each resulting in a 1 to 6 dilution. By the eighth tube there was less than one infectious unit ascribable to the initiating RNA, and the 15th tube contained less than one strand of the initial input. Nevertheless, every tube showed an increment in infectious units corresponding to the radioactive RNA found.

Figure 6 compares cumulative increments with time in newly synthesized RNA and infectious units. The agreement between the increments in synthesized RNA and newly appearing infectious units is excellent at every stage of the serial transfer and continues to the last tube. Long after the initial RNA has been diluted to insignificant levels, the RNA from one tube serves to initiate synthesis of biologically competent RNA in the next. It is clear that every step and component necessary to complete the replication must be represented in the reaction mixture described.

(9) Prospects for the Resolution of the RNA Replicating Mechanism.—We may conclude this discussion with an assessment of the current status of the RNA replication problem and an indication of the direction of our present efforts.

It must be emphasized that the doubts raised (§ 4) about the ribonuclease-resistant structures (RS) concern only their function. The structures are real and their existence must ultimately be explained. Certain quantitative features of the time, kinetics of appearance, and proportion of input strand involved in “RS” are difficult to reconcile with a model which insists that they intervene between the initial template and final product. Further, ribonuclease-resistant structures are observed with purified replicase whenever it is functioning abnormally (e.g., with fragments or in the presence of Mn++). On the basis of these and other difficulties, we maintain that a decision cannot be made at present on whether the RS are replicative intermediates of unknown structure, nonreplicative intermediates of unknown function, or simply nonfunctional artifacts.

The unambiguous analysis of a replicating mechanism demands evidence that the reaction being studied is in fact generating replicas. Ultimately, therefore, proof must be offered that the polynucleotide product contains the information necessary for the production of the corresponding virus particle in a suitable test system. The experiments described demonstrate that this rather rigorous requirement has finally been satisfied.

It should now be possible to study RNA replication in a simple system consisting of purified replicase, template RNA, riboside triphosphates, and magnesium. However, this is a necessary condition, not one sufficient for success. Possession of an enzyme of this sort does not, of itself, guarantee that any results observed are necessarily relevant to the nature of the replicating reaction. Attempts at the analysis of the replicating mechanism must recognize the implications of the fact that the enzymes involved are likely to be complicated molecules. High levels of complexity provide the flexibility which permits the occurrence of abnormalities, a potentiality which can be accentuated by exposure to either strange environments or unusual components. Thus, in the absence of primer, the DNA polymerase eventually initiates the synthesis of an AT-copolymer. In the presence of Mn++, the same enzyme will incorporate riboside triphosphates into a mixed polymer. Analogously, the DNA-dependent RNA polymerase synthesizes poly A if supplied only with ATP, a reaction which is inhibited if the other riboside triphosphates are added. Again, if presented with a single-stranded DNA, the transcriptase
synthesizes a DNA-RNA hybrid and if the template is RNA, a duplex RNA results. The fact that such variations from the norm can occur makes it difficult to draw incontestable conclusions from the appearance of a product in a reaction. Thus, for example, as will be detailed elsewhere, replicate makes an RNase-resistant structure if presented with either fragments of its own genome or intact heterologous RNA. We recognize that the abnormal has often been fruitfully used in the study of the normal and that even artifacts can ultimately serve to illuminate the reaction in which they are generated. However, it is first necessary to identify the normal. We insist, therefore, that in the test tube even more than in the cell, evidence other than mere existence must be provided before a component found is accepted as a normal intermediate of the replicative process.

The study of the normal functioning of the replicases described requires intact homologous RNA and the avoidance of Mn++. Furthermore, even under optimal conditions, as we know them, prolonged functioning of these enzymes in the enzymologist's test tube can create the possibility of accumulating abnormalities. Since the enzyme reaction described here does in fact produce RNA strands biologically indistinguishable from the input templates, it should be possible to test all the implications of any proposed mechanism. If two enzymes are required, both must be present and it should be possible either to establish their existence or to prove that one is sufficient. If an intermediate replicating stage intervenes between the template and the identical copy, then these forms should be demonstrably present in the reaction mixture. All experiments designed to test these alternatives must be continually monitored for biologically active product to ensure that the normal reaction is being followed.

A rather strong negative conclusion can be drawn from the data summarized concerning the possible role of transcriptase as the "second enzyme" for RNA replication, a mechanism some find attractive. The complete absence of detectable transcriptase from our preparation would appear to eliminate it as a participant in RNA replication.

It seems likely that the most telling data are derivable from experiments in which the initiation of new chains is synchronized. To begin with, the examination of the product synthesized, prior to the appearance of mature strands, can be compared with that formed in more extensive synthesis. The use of different isotopes on template (e.g., H³) and product (e.g., P³²) permits a sensitive search for intermediate complexes between the two, a prediction of the φX-174 model.

We may briefly list potentially informative experiments which use these and other devices:

1. There might be a comparison of ribonuclease resistance of product and template at various stages of synthesis.
2. A search could be made for a physical complex between the P³² product and the H³ template in sucrose gradients and in equilibrium density gradients of CsSO₄. In the latter the templates can, in some cases, be additionally labeled with N¹⁵ to give them a unique density position. Here the early (1–5% synthesis) events are most crucial.
3. A detailed analysis could be made of the base composition during the progress of early synthesis. The resulting data are particularly informative in the
case of Q3, since its A/U ratio is 0.75 and that of its complement is, therefore, 1.33. Consequently, the formation of the complement as an initial step is easily detected.

(4) Along similar lines, a comparison of nearest-neighbor analysis to all four bases in early and late synthesis should reveal whether a complement or the identical copy is being made in the early periods.

(5) The degree of complementarity between the product and the original template at various stages of strand formation could be determined by hybridization tests. In this connection, it may be noted that the required annealing experiments are not as simple, either logically or technically, as some recent contributions would suggest.

(6) The involvement of replicating complexes or complementary strands might not be detected by any of these experimental devices if they pre-existed in the enzyme preparations, either free or associated with active enzyme molecules. Here, however, advantage can be taken of the size (2 x 10^6 for RF and 1 x 10^6 for the complementary RNA) and the density of RNA or RNA-enzyme complexes. Enzyme can be isolated pycnographically in a density gradient at a density characteristic for nucleic acid-free protein, followed by characterization for size in a sucrose gradient. If the resulting enzyme is active and still completely satisfied by viral RNA, pre-existing complements or duplexes can hardly be invoked to explain their properties.

Virtually all the experiments listed above have been carried out and a few are in the final stages of completion. The detailed data and conclusions will be recorded elsewhere. Here we may state that, thus far, we have found no evidence to encourage the idea that a duplex containing the mature strand and its complement plays a role in replication.

It is important to emphasize that none of this should be taken to mean that our experiments have eliminated the use of complementarity in RNA replication. There are readily designed mechanisms which involve complementarity without requiring the synthesis of an intermediate duplex or the complete complementary strand. An extreme example may be briefly noted: Consider the possibility that a representative of each of the four nucleotides is attached to the enzyme. These could be permanent components or replaceable ones and are used by the enzyme for complementary reading of the template as a guide, via "allosteric instruction," for building an identical copy. Other mechanisms involving transient partial complements can also be devised.

It seems likely that many of the uncertainties which still exist about RNA replication will yield relatively soon to the proper experiments. We are tempted to end the present discussion with only a slight modification of the conclusion used in an earlier essay on protein synthesis. "The crucial experiments have not yet been executed. However, the systems required for their performance are with us, or close to hand. The outlook is depressingly bright for the quick resolution of another interesting problem.”

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