FURTHER EXTRACELLULAR DARWINIAN EXPERIMENTS WITH 
REPLICATING RNA MOLECULES: DIVERSE VARIANTS ISOLATED 
UNDER DIFFERENT SELECTIVE CONDITIONS*

BY REUBEN LEVISOHN† AND S. SPIEGELMAN
DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA
Communicated May 9, 1969

Abstract.—Experiments are described which demonstrate that it is possible to 
isolate in vitro a variety of mutant RNA molecules which exhibit qualitatively 
distinguishable phenotypes. The results suggest that precellular evolution could 
have involved selective forces of previously unsuspected diversity and subtlety.

Suitable adjustment of the selective conditions leads to the isolation of variants 
optimally designed to compete successfully with the original viral nucleic acid. 
One of the properties that can be built into the variants is resistance to the pres-
ence of inhibitory analogues of the normal riboside triphosphates. Potentially, 
such variants could be used as antiviral devices in conjunction with the more usual 
chemotherapeutic agents.

Introduction.—Proof that purified Qβ-repliase1 catalyzes the synthesis of both 
normal2, 3 and mutant4 infectious Qβ-RNA established that the RNA is the in-
structive agent in the replicative process. The fact that the RNA molecule 
satisfies the operational definition of a self-duplicating entity generated the 
possibility of performing extracellular Darwinian experiments.

The first step in exploiting the inherent potentialities of this system was a 
serial transfer experiment which resulted5 in the selection of variant V-1. This 
mutant replicated some 15 times faster than Qβ-RNA and retained 550 of the 
3600 residues originally present in the parental molecules.

We then showed6 that purified Qβ-repliase can be initiated to synthesize 
copies by a single molecule of template. The resulting clone of descendants pos-
sessed evident advantages for sequence studies and in addition made possible the 
inception of an in vitro genetics of replicating molecules. In performing these ex-
periments, a new variant (V-2) was isolated which replicated faster than V-1. 
Thus, measurable RNA synthesis occurred in a 15-minute reaction when initiated 
with as little as 0.29 μg of V-2. However, more than 300 times as much is re-
quired with V-1. This phenotypic difference has been maintained over many 
transfers.

The experiments thus far described were concerned with the isolation of 
mutants possessing increased growth rates under standard conditions. We here 
turn our attention to a question of no little theoretical and practical interest and 
inquire whether other mutant types can be isolated. In effect, we are asking the 
following question: Can qualitatively distinguishable phenotypes be exhibited 
by a nucleic acid molecule under conditions in which its information is replicated 
but never translated? The results to be reported show that numerous differenti-
able variants can be isolated, the number depending on the ingenuity expended in 
designing the appropriate selective conditions.
Materials and Methods.—All quantities are expressed per 0.125 ml standard reaction mixture.

(a) Standard reaction mixture: 10.5 μmoles Tris-HCl pH 7.4, 2.0 μmoles MgCl₂, 0.375 μmole EDTA (ethylenediamine tetraacetic acid), 100 μmoles each of ATP, CTP, GTP, UTP, and 20–40 μg Qβ-replicase. One of the nucleoside-triphosphates added was radioactively labeled with either H³¹ in the base or P³² in the α-phosphorus. Deviations from standard reaction mixture will be noted and involve lowering the concentrations of one of the four nucleoside triphosphates. All incubations were at 38°C.

(b) Enzyme: Qβ-replicase purified⁴ twice on DEAE was used as described previously.⁶

(c) Gel electrophoresis: Electrophoresis through 3.6% preswollen 0.9 × 6.0 cm bis-acrylamide cross-linked polyacrylamide gels was carried out for 2 hr at 10 mA/gel as detailed previously.⁷ Slices of 0.5 mm were made from frozen gels dried, dissolved for 6 hr at 80°C in 30% H₂O₂ and counted in liquid scintillation fluid.

(d) Base ratio: The determinations were performed on purified “plus” strands⁸ following a reaction in which all four nucleoside-triphosphates were equally labeled with H³¹ in the α-phosphorus. After alkaline hydrolysis the mononucleotides were separated by paper electrophoresis and determined according to Sanger et al.⁹

(e) Purification of variant RNA: Reactions were stopped by a fivefold dilution into a mixture of: 0.01 M Tris pH 7.4, 0.2 M NaCl, 0.003 M EDTA, and 0.2% SDS (sodium dodecyl sulfate). This was followed by two phenol extractions and three alcohol precipitations.

(f) Selection and isolation of variants: A reaction product of a previously isolated variant was inoculated at a concentration of 0.065 μg/0.125 ml into a reaction mixture of the specified selective medium. During incubation at 38°C, samples were taken out periodically, a portion being diluted at least 100-fold in 0.01 M Tris-HCl pH 7.4 + 0.003 M EDTA and frozen, and another aliquot assayed for cold trichloroacetic acid (TCA)-insoluble material.

Variants were isolated in the course of serial transfer experiments as modified by Leisohn and Spiegelman.⁵ Each transfer employed diluted product of the reaction just completed in which 0.075–0.15 μg of RNA had been synthesized. The dilution factor between transfers was gradually increased to 1 × 10⁴. Finally, the product of the last transfer was cloned.⁶

Results.—“Nutritional” mutants: (a) Isolation: One rather general approach for obtaining a variety of mutants is to run the syntheses under less than optimal conditions with respect to a component or parameter of the reaction. If a variant arises which can cope with the imposed suboptimal situation, continued transfer should lead to its selection over wild type. We now describe an example of how this can be done with variations in the levels of the riboside triphosphates.

As may be seen from Figure 1, the rate of synthesis of V-2 begins to decrease sharply as the level of CTP drops below 20 μmoles per 0.125 ml. At a CTP

![Fig. 1.—CTP concentration curve. Reaction mixtures of 0.125 ml containing the indicated concentrations of CTP and otherwise identical to "standard reaction mixture" were incubated for 30 min at 38°C with 20 μg Qβ-replicase and 0.01 μg variant-2 RNA. Subsequently, the acid insoluble radioactivity was determined. In the "complete medium" (containing 100 μmoles CTP) 1.0 μg of V-2 RNA were synthesized.](image-url)
concentration of 2 mumoles, the rate of synthesis of V-2 is only 25 per cent of normal. At 1 mumole of CTP the rate decreases to 5 per cent of normal.

With this information available, a search was made for variants which could replicate better than V-2 on limiting levels of CTP. A serial transfer experiment at 2 mumoles of CTP per reaction was initiated with Q8-RNA, culminating after ten transfers with the appearance of V-4. A second series of transfers at 1 mumole of CTP per reaction was then started with V-4 and after 40 transfers led to the isolation of V-6.

Figure 2 describes the replication of variants 2, 4, and 6 in limiting CTP (1 mumole per 0.125 ml). The slopes of the semilog plots permit an estimation of the doubling times during logarithmic increase as 1.81 minutes for V-2, 1.41 minutes for V-4, and 1.16 minutes for V-6. Evidently both V-4 and V-6 possess a heritable feature which permits them to overcome the disadvantages imposed by the low level of CTP.

(b) The basis of the mutant phenotype: The fact that variants 4 and 6 replicate 28 per cent and 56 per cent better, respectively, than V-2 at low levels of CTP might be explained on the basis of smaller sizes or modification of base composition towards a lower cytosine content.

Figure 3 compares the sizes of V-2 and V-6 on polyacrylamide gels and shows that there is no significant difference in chain length. In passing, it may be noted that similar comparisons revealed very little change in length in any of the variants thus far selected.

Table 1 compares the base composition of V-2 and V-6. Here again, no significant differences are detectable. It should be noted that the sensitivity of this test is of the order of 0.5 per cent and changes involving a small number of residues would be difficult to detect.

It is evident that the modifications leading to the properties possessed by variants 4 and 6 do not involve massive modification in the composition of the molecule. The identification of the changes will require more subtle examinations such as oligonucleotide fingerprint patterns, and these are being carried out.

The fact that the most obvious pathways for solving the problem of low CTP were not employed leads one to consider more sophisticated devices for achieving the desired end result. It is useful here to recall that the mutant RNA molecules
must complex with the replicase. Thus, changes of sequence which would leave such gross features as base composition and size unchanged could, nevertheless, lead to different secondary structures of the mutant molecules. These in turn could have allosteric effects on the replicase, permitting the complex to employ CTP more effectively at suboptimal concentrations. If this were the case, and if there were a common site for the four riboside triphosphates analogous to the DNA polymerase,¹⁰ it might be expected that a mutant selected for better replication on low CTP would also exhibit increased capacities to accommodate to low levels of the other riboside triphosphates.

Table 2 summarizes data comparing the logarithmic synthesis of variants V-4 and V-6 with that of V-2 on limiting levels of each of the four riboside triphosphates. The data show that the two variants selected on low CTP also do much better on limiting concentrations of the other three substrates. More definitive delineation of the underlying mechanism will require binding studies of substrates with enzyme complexed to mutant and wild-type templates.

(2) Selection of a variant resistant to an inhibitory analogue: Tubercidin is an analogue of adenosine in which the nitrogen atom in position 7 is replaced by a carbon atom. Tubercidin triphosphate (TuTP) inhibits the synthesis of Q8-RNA in vitro (Alan Kapular, personal communication). TuTP cannot completely replace ATP in the reaction. It is clear, however, from the following indirect experiment that it is incorporated into the growing chains. A series of reactions

TABLE 1. Base ratios of variants.

<table>
<thead>
<tr>
<th>Base</th>
<th>V-2</th>
<th>V-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>24.8</td>
<td>24.8</td>
</tr>
<tr>
<td>A</td>
<td>23.2</td>
<td>23.5</td>
</tr>
<tr>
<td>G</td>
<td>26.6</td>
<td>26.7</td>
</tr>
<tr>
<td>U</td>
<td>25.4</td>
<td>25.1</td>
</tr>
</tbody>
</table>

The base composition of purified plus strands of variant 2 and variant 6 were determined as described in Methods. The numbers represent mole per cent.
TABLE 2. Logarithmic synthesis of variant RNA on limiting media.

<table>
<thead>
<tr>
<th>Limiting nucleotides</th>
<th>M₅moles</th>
<th>Variant</th>
<th>Doubling time (min)</th>
<th>Relative Slope Compared to V-2</th>
<th>Compared to V-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>2</td>
<td>0.42</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>4</td>
<td>0.42</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>0.35</td>
<td>1.21</td>
<td>1.21</td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
<td>2</td>
<td>2.41</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>4</td>
<td>1.54</td>
<td>1.56</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.47</td>
<td>1.64</td>
<td>1.05</td>
</tr>
<tr>
<td>CTP</td>
<td>1</td>
<td>2</td>
<td>1.81</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>4</td>
<td>1.41</td>
<td>1.28</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.16</td>
<td>1.56</td>
<td>1.21</td>
</tr>
<tr>
<td>GTP</td>
<td>9</td>
<td>2</td>
<td>3.05</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>4</td>
<td>2.25</td>
<td>1.35</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>2.31</td>
<td>1.31</td>
<td>0.97</td>
</tr>
<tr>
<td>UTP</td>
<td>2</td>
<td>2</td>
<td>2.06</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>4</td>
<td>1.69</td>
<td>1.22</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.54</td>
<td>1.33</td>
<td>1.09</td>
</tr>
</tbody>
</table>

The data are based on the experiment shown in Fig. 2 and similar experiments performed in standard reaction mixture and in reaction mixtures with only 4 M₅moles ATP, 9 M₅moles GTP, or 2 M₅moles UTP.

were run at increasing levels of TuTP in the presence of fixed amounts of P²³⁷-UTP and H³-ATP. The latter permits determination of the U to A ratio in the product. Figure 4 shows the outcome which indicates that TuTP can replace A but with a less than equal probability.

It was of some interest to see whether one could derive a mutant which would show resistance to the presence of this agent. In such experiments, it is desirable to have the ratio of the analogue to ATP as high as possible. To attain this more readily, a variant was isolated on limiting ATP concentration. Variant 6 was chosen to start a series of transfers in a reaction mixture containing 1.5 M₅moles of ATP, and this led to the isolation of V-8. The doubling time of V-8 in the reaction mixture with 1.5 M₅moles of ATP was 2.8 minutes as compared with 8.4 minutes for V-6, the starting variant.

The replication rate of V-8 on a reaction mixture containing 5 M₅moles of ATP was inhibited fourfold upon the addition of 30 M₅moles of TuTP. A serial transfer was initiated with V-8 on the inhibitory medium and led to the isolation of V-9. The doubling time of V-9 in the presence of TuTP was 2.0 minutes as compared with 4.1 minutes for V-8. In the absence of TuTP, both variants are synthesized with a 1.0-minute doubling time. It is clear that V-9 exhibits a specifically increased resistance to the inhibitory effect of TuTP. The resistance mechanism does not involve a more effective exclusion of TuTP as measured by an experiment similar to that described in Figure 3. Thus, at 30 M₅moles of TuTP and 5 M₅moles of ATP, the ratio of U to A in the product was 3.6 for V-8 and 3.5 for V-9, the resistant mutant.

Discussion.—Table 3 lists the variants isolated in the experiments described and summarizes the relevant information on their origins and conditions of selection. It will be noted that V-4 is an independent derivative from the parental Q₂³ RNA. Another variant V-3 (not listed) was isolated with limiting CTP starting with V-2 instead of Q₂³ RNA. V-3 possesses phenotypic properties
Fig. 4.—Substitution of ATP by TuTP. Synthesis of RNA templated by 0.001 μg V-3 RNA in the presence of 40 μg Q3-replicase was allowed to take place for 40 min in a reaction mixture containing 5 mpmoles ATP, the indicated amounts of TuTP, and H3-ATP and P32-UTP. The U to A ratio in plus strands of variant is 6/5, which we may take as unity. If tubercidin can replace adenosine with equal probability, the U to A ratio in the product should vary with the ratio of TuTP to ATP in the reaction mixture in the manner described by the upper dashed curve (labeled equivalent substitution). If there is no substitution of adenosine by tubercidin, the ratio of U to A should remain normal and independent of the relative amounts of TuTP present (lower dashed curve labeled no substitution). The unbroken line indicates the actual incorporation ratio corrected for an input of 6 × 10^6 cpm/100 mpmoles P32-UTP and 5 × 10^6 cpm/100 mpmoles H3-ATP.

Table 3. Conditions used in isolation of variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Selective limitations</th>
<th>RNA used to start selection</th>
<th>No. of transfers at dilution of 1.25 × 10^4</th>
<th>Total no. of transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-26</td>
<td>None</td>
<td>Q3</td>
<td>...</td>
<td>17</td>
</tr>
<tr>
<td>V-4</td>
<td>2 mpmoles CTP</td>
<td>Q3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>V-6</td>
<td>CTP</td>
<td>V-4</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>V-8</td>
<td>1.5 &quot; ATP</td>
<td>V-6</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>V-9</td>
<td>5 &quot; ATP</td>
<td>V-8</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>+30</td>
<td>TuTP</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Variants were selected on standard reaction mixture, or on a standard reaction mixture modified to contain one of the four nucleoside triphosphates at the indicated concentration. Starting with the RNA's indicated in column 3, a series of transfers were made with reaction product, diluted 1.25 × 10^4-fold, as detailed in Methods. Subsequently, the dilution factor between transfers was gradually increased to about 1 × 10^8.

indistinguishable from those of V-4. Thus, one can arrive at the V-4 phenotype either from Q3-RNA or from V-2. It seems probable that Q3-RNA passes through the V-2 stage before arriving at the V-4 phenotype.

The comparatively conservative nature of the replicative process is illustrated by the virtual identity of base compositions of V-2 and V-6 seen in Table 1. These two mutants are independent isolates and are separated by two lengthy and severe selections on limiting CTP. No reflection of this is seen in the base compositions of the two.
It will be of enormous interest to compare the actual sequence changes among the mutants differing in their relatedness and phenotypic properties. With this information available, one can begin to construct the probable secondary structure modifications. Only then will we be in a position to begin the attempt to understand the molecular basis of these new phenotypes.

We pointed out previously that extracellular Darwinian selections may mimic one aspect of precellular evolution, i.e., when environmental selection operated only on the replicating gene and not on the gene product. Such experiments provide some insight into the rules of these early stages of evolution. It was not a priori obvious what kinds of selective forces could be operative since much depended on how many different ways a molecule could be selected as superior by the environment. The experiments reported here reveal an unexpected wealth of phenotypic differences which a replicating nucleic acid molecule can exhibit. It is true that many of these involve interaction between nucleic acid molecules and a highly evolved protein catalyst. However, it is possible to imagine similar types of interactions with a primitive surface catalyst. Sequence changes which would increase slightly the catalytic effectiveness could have powerful selective effects in these precellular stages of evolving genetic material.

It is apparent from the limited number of examples described that a host of new mutant types possessing predetermined phenotypes can be isolated by varying other parameters of the system. In addition, one can expand the possibilities by introducing initially neutral agents (e.g., proteins) with which the replicating molecules may interact. Selection can then be exerted to favor variants that can induce these foreign agents to become participants in the replicative process.

Finally, we should like to note a practical implication of the mutant resistant to the inhibitory analogue TuTP. We pointed out earlier that these abbreviated variants possess a number of features which make them potentially powerful tools as chemotherapeutic agents. They combine a very high affinity for the replicase and a rapid growth rate. They compete effectively with the normal viral nucleic acid for the replicase and thus halt the progress of virus production. To these features we can now add a third, namely, resistance to a chemotherapeutic agent effective against the original virus particle. All these features can be built into one variant by the kinds of serial selections described here. This adds another dimension to the potential use of these agents as chemotherapeutic devices.

* This investigation was supported by U.S. Public Health Service research grant CA-01094 from the National Cancer Institute and the National Science Foundation.
† Recipient of a Damon Runyon Cancer Research Fellowship.
1 Haruna, I., and S. Spiegelman, these PROCEEDINGS, 54, 579 (1965).
3 Pace, N. R., and S. Spiegelman, these PROCEEDINGS, 55, 1608 (1966).
6 Levisohn, R., and S. Spiegelman, these PROCEEDINGS, 60, 806 (1968).