Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 60, No. 3, pp. 866-872. July, 1968.

*THE CLONING OF A SELF-REPLICATING RNA MOLECULE**

BY REUBEN LEVISOHN† AND S. SPIEGELMAN

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

Communicated May 9, 1968

The availability of purified[1] Qβ-replicase,[2] which can mediate virtually unlimited synthesis of biologically active[3] and infectious viral RNA, has made possible a variety of informative investigations. The use of a temperature-sensitive mutant led to the demonstration that the RNA molecule is the instructive agent in the replicative reaction, hence the rigorous conclusion[4] that the *in vitro* synthesis of a self-replicating molecule had in fact been achieved.

Among the interesting possibilities thus generated was the feasibility of performing Darwinian experiments in which the replicating molecules are exposed to a variety of sclection pressures. Mills, Peterson, and Spiegelman[5] reported experiments of this nature designed to select molecules that could replicate faster than the original viral RNA. During the course of the serial transfers involved, variants of decreasing length made their appearance sequentially. The mutant isolated after the 74th transfer replicated some 15 times faster and contained only 550 of the 3600 residues originally present in the parental Qβ-RNA.

We shall now focus attention on the use of this system to generate clones descended from individual RNA strands. Such experiments possess an inherent historical interest since the successful cloning of a molecule replicating *in vitro* has not, thus far, been recorded. More important, however, is the fact that the resulting clones provide the sort of uniformity required for sequence studies of variant molecular structures. The same experiments can also provide useful information on molecular weights and genetic stability of distinguishable variant molecules of independent origin.

The approach used depends on a straightforward comparison of the observed frequency distribution with that expected from Poisson statistics in a series of repeated syntheses. Thus, *if one strand is sufficient to start a synthesis*, then the proportion of tubes showing no synthesis should correspond to e^{-m}, m being the average number of strands inoculated per tube. Further, if the onset and syntheses were adequately synchronized, one might hope to identify tubes that received one, two, or three strands; these should appear with frequencies corresponding to me^{-m}, $(m^2/2!)e^{-m}$, and $(m^3/3!)e^{-m}$, respectively.

It is evident that the contemplated experiments make rather severe demands on the purity of the replicase preparation employed. It must be sufficiently free of contaminating nucleases so that there is a high probability that a single strand will initiate and complete its replication. Further, the content of contaminating RNA must be low enough so that tubes that receive no added template molecule will not show evidence of synthesis in the time period of the experiment. Note that as little as 1 $\mu\mu$g of residual RNA in 10 μg of enzyme (i.e., 1 in 10^7 contamination by weight) would correspond to the presence of 1.2×10^7 strands. This difficulty can be obviated with the use of a variant Qβ-RNA that

grows much faster than the ordinary Qβ-RNA molecules expected as contaminants; this procedure was followed in the experiments to be described.

It is the purpose of the present paper to detail the relevant experiments. The data obtained show that the necessary prerequisites can be met and that it is in fact possible to produce clones of variant-RNA molecules.

Materials and Methods.—(a) *Enzyme preparations:* Qβ-replicase preparations were purified from Qβ-infected *Escherichia coli* Q13 cells according to Haruna and Spiegelman,[2] except that the purification on an *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) column was done twice. The enzyme preparations used were checked repeatedly for absence of "background reaction," i.e., synthesis of RNA without addition of external template.

(b) *Assay of enzyme activity:* The procedure of Spiegelman *et al.*[3] was used with slight modifications. The standard replicase reaction mixture included, in addition to the components used previously, 0.75 μmole ethylenediaminetetraacetate (EDTA) and an additional 0.75 μmole magnesium chloride per 0.25 ml. Unless otherwise specified, each reaction had a total volume of 0.125 ml, containing 20 μg Qβ-replicase, and was incubated for 15 min at 38°C. α-P^{32}-uridine 5′-triphosphate (UTP) or α-P^{32}-guanosine 5′-triphosphate (GTP) was synthesized and used to monitor incorporation into acid-insoluble (5% trichloroacetic acid (TCA)) polynucleotide as described previously.[6]

Unless specified otherwise, results are expressed in terms of standard reaction mixtures with an input of 10^6 cpm P^{32}-UTP or P^{32}-GTP per 0.25 ml after subtraction of background counts. At this value, incorporation of 4100 cpm of P^{32}-UTP and 4150 cpm of P^{32}-GTP are equivalent to the synthesis of 1 μg of variant RNA.

(c) *Gel electrophoresis:* As detailed previously,[7] electrophoresis through 3.6% preswollen, 0.9 × 4.5 cm, *bis*-acrylamide cross-linked polyacrylamide gels was carried out for 2 hr at 10 ma/gel. After the gels were frozen, 0.5-mm slices were made with a precooled microtome. These were washed with 4% TCA, dissolved for 6 hr at 80°C in 30% H_2O_2, and counted in liquid scintillation fluid.

(d) *Ultraviolet irradiation:* A solution of 7 μg/ml Qβ-RNA was irradiated with a 15-watt Champion germicidal ultraviolet lamp from a distance of 142 mm for 7 min (to be described elsewhere.[8]).

Results.—(a) *Selection of variant-2 RNA molecules:* In the original serial transfer experiments,[5] which led to the isolation of variant-1 (V-1), the product of each reaction was diluted 12.5-fold in the course of being used as a template for the next tube. To maintain the selection pressure, the period of incubation was shortened at intervals.

To isolate a new fast-growing mutant for the present investigation, a modification was introduced in the selection procedure. The incubation interval at 38°C was held constant at 15 minutes and increasing selection pressure was achieved by recurrent sharp increases in the dilution experienced by successive transfers. This variation was employed for several reasons. First, it was interesting to see whether a fast-growing mutant, distinguishable from V-1, would emerge. Further, this type of selection could increase the severity and extent of the selection pressure. Finally, the use of very low initiating inputs would more closely mimic the kind of experiments required for the Poisson analysis. Consequently, the mutant finally evolved would be more suitable for experiments of this nature.

The Qβ-RNA used as the initial template was subjected to ultraviolet irradiation as described in *Materials and Methods.* This treatment is not essential but was included to accelerate the rate of mutant production. Experiments with

unirradiated RNA yielded similar mutants. The first reaction was initiated by the addition of 0.07 μg RNA, 0.01 ml being transferred to the next tube. This 125-fold dilution transfer was continued for five tubes, at which point a fast-growing mutant had already arisen. In the next seven transfers, 0.01 ml of a 1×10^6-fold dilution of each reaction was used, corresponding to approximately 1×10^5 strands. In the final five transfers, 0.01 ml of dilutions ranging from 1×10^7-fold to 2.5×10^{10}-fold were employed. The variant RNA that evolved by the 17th transfer was selected for more detailed study and will be called variant-2 (V-2).

(b) *Comparison of V-1 and V-2:* It was interesting to compare the properties of V-1 and V-2, particularly in their response to low inputs of template. Figure 1 and Table 1 show the behavior of the two variants in a 15-minute incubation at 38°C. V-2 is clearly superior at initiating levels below 100 $\mu\mu\mu$g of RNA.

Figure 2 compares the growth kinetics of the two variants during the arithmetic (*A*) and exponential (*B*) growth phases. During the exponential period, the doubling time of V-2 was 0.403 minute as compared with 0.456 minute for V-1. With this difference in growth rate, V-2 would experience four more doublings (i.e., a 16-fold increase) than V-1 in a 15-minute period of logarithmic growth.

In view of the different rates of synthesis, it was interesting to make a size comparison. The two variants were radioactively labeled, one with H^3, the other with P^{32}, and coelectrophoresed in the same gel. The results shown in Figure 3 indicate that the double-stranded intermediates (*first peak*) and the single strands (*second peak*)[5] all fall in the same region of the gel. The barely detectable difference in size is not enough to account for the higher growth rate of V-2.

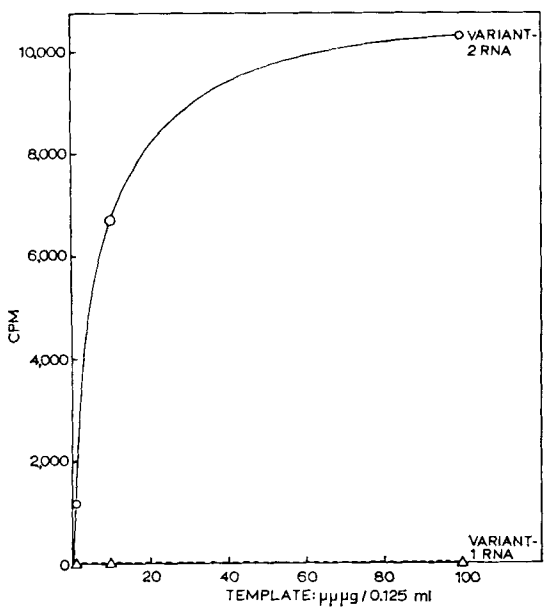

FIG. 1.—Comparison between the synthesis of V-1 RNA and V-2 RNA when used as templates at very low concentrations. The indicated concentrations of V-1 RNA and V-2 RNA were added as templates to 0.125 ml of standard reaction mixture. Following a 15-min incubation at 38°C, the P^{32}-UTP incorporation into acid-insoluble material was determined as described in *Materials and Methods*.

TABLE 1. *RNA synthesis initiated by V-1 and V-2.*

Initial concentration of template ($\mu\mu\mu$g/0.125 ml)	V-1 (cpm/0.25 ml)	V-2 (cpm/0.25 ml)
1	0	1,180
10	0	6,700
10^2	0	10,300
10^3	5,850	16,700
10^4	11,300	18,800
10^5	13,200	20,100
10^6	17,000	22,400

Conditions are the same as in Fig. 1.

(c) *Cloning of V-2:* Like V-1,[5] V-2 has a molecular weight of about 177,000, corresponding to approximately 550 residues; thus one strand is equivalent to 0.29 $\mu\mu\mu$g of RNA.

A reaction was run to synthesize 13.2 μg of P^{32}-labeled V-2 RNA as described in *Materials and Methods.* The product was diluted in a standard $Q\beta$-reaction mixture to 2.9 $\mu\mu\mu$g/ml, and 0.1 ml was placed in each of 82 tubes. Thus, each tube received, on the average, one strand of template. All these steps were carried out at 0°C, at which no detectable synthesis occurs. The rack of tubes was then placed in a 38°C water bath and held there for 30 minutes. The reactions were stopped and the amount of radioactive polynucleotide synthesized in each tube was determined (see *Materials and Methods*). The results are summarized in Table 2.

If the assumptions underlying the Poisson distribution have been satisfied by the conditions of the experiment, one would expect 36.8% of the 82 tubes (i.e., 30.2) to show no synthesis. This is in excellent agreement with the 30 tubes found.

It was interesting to see whether among the tubes exhibiting synthesis one could distinguish those initiated by one strand from those that received two or

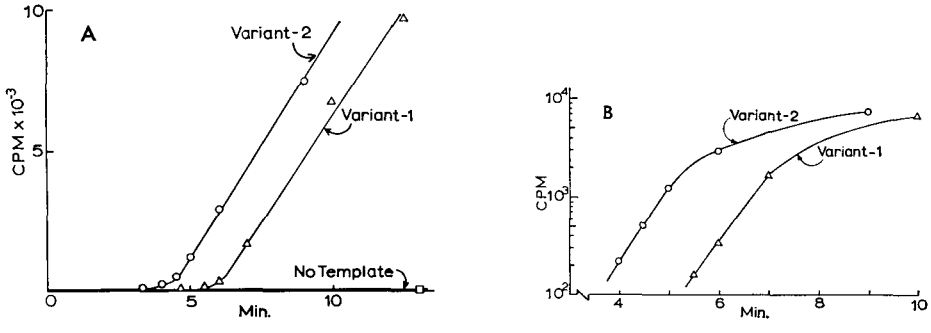

FIG. 2.—Kinetics of RNA synthesis initiated by V-1 and V-2 RNA. Standard reaction mixtures containing 0.0001 μg variant RNA per 0.25 ml were incubated at 38°C. Samples of 0.05 ml were withdrawn at the indicated times to determine the incorporation of P^{32}-UTP into acid-insoluble material. The actual counts per sample were 14 times higher than the standardized counts (see *Materials and Methods*), which were plotted on an arithmetic scale (*A*) and a semilogarithmic scale (*B*).

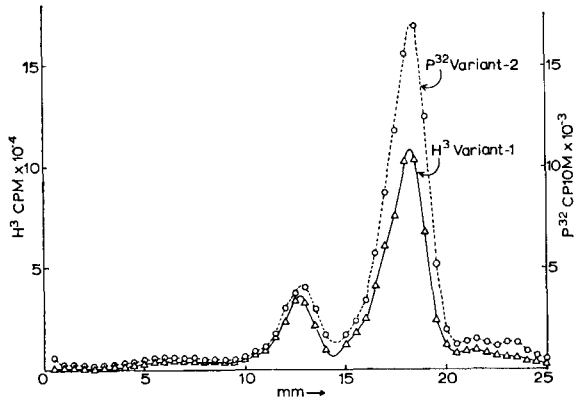

FIG. 3.—Gel electrophoresis of V-1 and V-2. Standard reaction mixtures of 0.125 ml initiated with 0.2 μg of V-1 or 0.01 μg of V-2 and labeled with about 1.5×10^7 cpm H^3-ATP or 4×10^6 cpm P^{32}-UTP, respectively, were incubated for 20 min at 38°C. The reactions were terminated with 0.01 ml of 2.5% sodium dodecyl sulfate. Portions of the two reactions were mixed and electrophoresis through polyacrylamide gel was carried out as in *Materials and Methods*.

more. To achieve the desired identification, the following calculation was performed. The total experiment involved the functioning of 82 templates. The sum of counts observed in all tubes (25,340 cpm after correction for controls without added RNA, which was 22 cpm/tube) is a measure of the total RNA initiated by all 82 templates. If the sum is divided by 82, one obtains 309 cpm as the average amount ascribable to a single template. The actual incorporation observed in each tube can then be divided by 309 and the result rounded out to the nearest integer to yield an approximation of the number of strands that initiated the synthesis in that tube. The resulting data can then be compared with that expected, as is done in Table 2. The average and the corresponding deviation (2σ) for each group are also listed. One finds here a remarkable agreement between what was found and what was expected from a Poisson distribution of template strands in the 82 tubes. The chances are clearly excellent that tubes that exhibited incorporation close to the average expected for one strand (309 cpm) were in fact initiated by single strands.

As a further check on the validity of this sort of approximation, a modification of the original Poisson experiment was performed, in which the *average input was varied*. Three sets of 30 tubes each were set up as described for Table 2. Each set was seeded with a different average number of initiating strands ranging

TABLE 2. *Distribution of template strands at an average input of one template strand per tube.*

Strands per tube	Polynucleotide (r)	Among 82 Tubes Expected	Among 82 Tubes Found	Average ± 2σ (cpm/tube)
0	0.368	30.2	30	17 ± 76
1	0.368	30.2	29	321 ± 173
2	0.184	15.1	19	613 ± 184
3	0.0613	5.4	3	841 ± 58
4	0.0153	1.3	1	1334

A reaction mixture in which 13.2 μg of V-2 RNA were synthesized was diluted to a final concentration of 2.9 $\mu\mu$g/ml RNA in a standard reaction mixture containing 7.8×10^5 cpm P^{32}-GTP per 0.25 ml. Each of 82 tubes received 0.1 ml. Following a 30-min incubation at 38°C, the acid-insoluble P^{32}-GTP was determined as described in *Materials and Methods*. The average is for the actual number of counts (not standardized) after subtraction of the average of nine control tubes (22 cpm) to which no variant RNA was added. In this experiment, 3240 cpm is equivalent to 1 μg of variant RNA.

TABLE 3. *Distribution of template strands at an average of one, two, or three template strands per tube.*

(r) Strands per tube	Average Input: One Strand Found	One Strand Expected	Two Strands Found	Two Strands Expected	Three Strands Found	Three Strands Expected
0	13	11.0	3	4.1	2	1.5
1	9	11.0	9	8.1	2	4.5
2	6	5.5	13	8.1	7	6.7
3	1	1.8	2	5.4	6	6.7
>3	1	0.7	3	4.3	13	10.6

V-2 RNA was diluted to final concentrations of 2.9, 5.8, and 8.7 $\mu\mu\mu$g/ml RNA in a standard reaction mixture containing 80 μg/0.25 ml Qβ replicase. Three sets of 30 tubes each were set up. Each set received 0.1-ml portions of one of the three reaction mixtures and was then incubated for 25 min at 38° and treated as in Table 1.

from one to three. The results are summarized in Table 3. Again, the agreement between the observed and expected at each average input is excellent.

Discussion.—It is apparent from the results described that the replicase system has been sufficiently freed of interfering reactions to permit initiation of synthesis by a single strand of template. Thus, the generation of clones of descendants from an individual RNA molecule was made possible. It is of interest and no little convenience that one can actually identify, by the amount of RNA synthesized, those tubes that were initiated by a single strand. It is not, however, necessary to depend on this property of the system. One can start with a dilution such that the vast majority of the tubes receive no templates. Under these conditions, those that do receive templates are very unlikely to receive more than one.

We may note here another method of cloning that is, in principle, possible. The enzyme and substrates could be incorporated into a semisolid medium (e.g., a soft agar layer), and the initiating strands spread on the surface. The resulting clones could then be located and picked much as one does with bacterial colonies.

The fact that 0.29 $\mu\mu\mu$g of RNA satisfies the Poisson expectation for an average of one strand immediately allows us to calculate the molecular weight as 174,000 daltons, which is in good agreement with that deduced from gel electrophoresis.

Needless to say, there is no guarantee that all individuals in a clone will be identical. They will be as similar as the mutation frequency will permit, which is as close as one can get with self-duplicating objects. In this connection, it should be noted that V-1 and V-2 have retained their phenotypic difference in growth rates over many transfers. This suggests the possibility of isolating and maintaining other mutant types, a possibility that has been realized in experiments recently performed.[9]

We have pointed out elsewhere[5] the potential usefulness of developing mutant molecules. Cloning them increases their utility for more detailed studies such as sequencing. It is also a necessary requisite for a "genetic" analysis of the replicating molecules.

Finally, a note of caution: the synthesis of self-duplicating molecules entails the risk of introducing them as laboratory environmental contaminants. As was seen in the experiments described, one molecule *can* take over a reaction.

This potential source of confusion has in fact been realized several times in our laboratory, beginning early in 1967 soon after we synthesized our first fast-growing mutant. The initial indication that we were being inconvenienced by our own creations was a sudden inability to prepare replicase exhibiting dependence on added template. In all instances, the difficulty was traced to contamination of a commonly employed assay reagent with a fast-growing variant. The unique phenotypic properties enabled us to identify it as one we had synthesized and isolated. These molecules are remarkably stable under a variety of conditions. As much care as is normally employed with bacteria and viruses must be exercised to exclude these molecules as unwanted intruders in experiments.

Summary.—Experiments have been described that show that purified Qβ-replicase can be initiated to synthesize copies of a mutant Qβ-RNA by a *single* strand of template. The resulting *clone* of descendants provides a population of individuals possessing the kind of uniformity required for sequence studies. Further, the fact that distinguishable clones can be isolated and maintained makes possible the inception of an *in vitro* genetics of replicating molecules.

* This investigation was supported by USPHS research grant no. CA-01094 from the National Cancer Institute and the National Science Foundation.

† Recipient of a Damon Runyon Cancer Research Fellowship.

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

[2] Haruna, I., and S. Spiegelman, these PROCEEDINGS, **54**, 579 (1965).

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

[4] Pace, N. R., and S. Spiegelman, *Science*, **153**, 64 (1966).

[5] Mills, D. R., R. L. Peterson, and S. Spiegelman, these PROCEEDINGS, **58**, 217 (1967).

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

[7] Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman, *J. Mol. Biol.*, **26**, 373 (1967).

[8] Levisohn, R., G. Weber, and S. Spiegelman, in preparation.

[9] Levisohn, R., and S. Spiegelman, in preparation.