

*CONSERVATION OF A VIRAL RNA GENOME DURING
REPLICATION AND TRANSLATION**

BY R. H. DOI† AND S. SPIEGELMAN

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA, ILLINOIS

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In organisms with a DNA genome three principle modes of information transfer are recognized and distinguished by the end purposes they serve. The first is duplication, designed to provide exact copies for hereditary transmission. The second is a transcription which converts the information into RNA complementary copies. Finally, we have a translation from the four-unit language of the nucleic acids to the twenty-element parlance of the proteins. The existence of viruses with RNA genomes raises obvious questions of the mechanisms employed to attain the same ends.

It has recently been shown¹ that neither before nor after infection can one detect sequences in the DNA of the host cell which are complementary to that of the viral RNA. These results suggest that the RNA viruses do not employ DNA as an informational component at any step unique to the production of mature virus particles. One is led therefore to predict a mechanism of RNA synthesis involving an RNA dependent polymerase. The available evidence makes it unlikely that an enzyme of this sort pre-exists in the uninfected cell. All recognized RNA components, including "informational,"^{2, 3} ribosomal,^{4, 5} and transfer RNA^{6, 7} have been shown to be complementary to sequences in homologous DNA. Their formation can therefore be adequately explained in terms of the DNA-dependent RNA polymerase.^{8, 9} This conclusion is further strengthened by the observation that actinomycin D inhibits normal cellular RNA synthesis but is unable to interfere with viral RNA formation.^{10, 11}

Considerations such as these lead to the prediction that the viral RNA injected into the host cell must contain the structural program for a new RNA polymerase. Since this enzyme must be synthesized *before* replication can begin, it follows that the entering RNA must *itself serve as a protein program and be conserved during its translation into protein.*

It is the purpose of the present paper to provide information pertinent to the prediction of conservation. For reasons which will become apparent it was technically simpler to answer the following more inclusive question: *Is the incoming strand conserved during translation and replication?* To identify the parental RNA, double labeling with N¹⁵ and P³² was used. Recovery of the original doubly labeled strand can be interpreted unambiguously as conservation only if the following possible complications can be eliminated: (a) the existence of unattached or reversibly at-

tached virus particles; (b) contamination of the sample analyzed with infected cells which did not produce virus components from the injected strands; (c) the presence of nonparticipating strands in cells which were multiply infected. Procedures will be described which avoid these sources of confusion. The data obtained are consistent with complete conservation of the RNA genome during all the replications and translations required for a lytic cycle.

Materials and Methods.—(a) *Cells, virus, and media:* The bacterial virus MS ϕ 2 was provided by Dr. Alvin J. Clark and is similar to the RNA bacteriophage (f2) described by Loeb and Zinder.¹² The phage was grown and assayed according to the procedures of these authors. Infectious centers were, as usual, assayed in the presence of MS ϕ 2-antiserum. The minimal medium (SC) routinely used is the same as that employed previously.² The general buffer used, designated by TM, is Tris at 0.01 *M* and 0.005 *M* Mg⁺⁺ buffered at pH 7.4.

(b) *Preparation of extracts from infected cells:* The lysozyme-freeze thaw method as detailed by Hayashi and Spiegelman² was used to prepare extracts which were cleared by centrifugation at 15 *g* for 15 min to yield a supernatant (15G-15S). Where pertinent these were analyzed in linear sucrose gradients for distribution of O.D.²⁶⁰ and radioactivity. In all cases acid precipitable radioactivity was assayed on millipore membranes in a Packard scintillation spectrometer as detailed previously.² *Ribonucleic acid preparation and purification:* All RNA preparations, from either mature phage or cell lysate, were purified by the phenol procedure.¹³ *E. coli* 23S RNA-H³ was prepared by the method of Yankofsky and Spiegelman.⁴

(c) *Cesium sulfate density gradient centrifugation:* The cesium sulfate was obtained from Penn Rare Metals, Inc., Revere, Pennsylvania, and recrystallized repeatedly from water until the optical density at 260 $m\mu$ of a 59% (w/w) solution was less than 0.1. The densities of ribonucleic acids in cesium sulfate were determined by the method of Hearst and Vinograd.¹⁴ For centrifugation in the preparative Spinco Model L ultracentrifuge, 2.1 ml of a cesium sulfate solution ($\rho = 1.88$), RNA solutions and 0.01 *M* phosphate buffer, pH 6.8, were mixed to a final volume of 3.0 ml. The final density of the solution was 1.617. The samples were centrifuged for 72 hr at 33,000 rpm at 25°C in the SW 39 rotor. Fractions of 0.061 ml were then collected from the bottom of the tubes. The refractive index of every fifth sample was determined to convert to density by means of a standard curve. The samples were then diluted and analyzed for O.D.²⁶⁰ and radioactivity.

(d) *Preparation of N¹⁵-P³²-labeled virus:* The growth medium contained: 0.055 *M* N¹⁵H₄Cl; 0.170 *M* NaCl; 0.054 *M* KCl; 0.006 *M* MgSO₄; 0.0005 *M* PO₄; 0.0006 *M* FeCl₃; 0.050 *M* Tris buffer, pH 7.2, 0.011 *M* glucose, 0.0002 *M* CaCl₂, 23.5 mc P³².

E. coli K-10 was added to 100 ml of medium to give an initial cell density of about 7×10^7 per ml and when it reached 2×10^8 , P³² was added. At 5×10^8 , the cells were infected at a multiplicity of 0.1 with MS ϕ 2. The culture was allowed to shake for 12 hr after infection.

At this time the cell suspension was centrifuged to separate the unlysed cells from the lysate. The unlysed cells were suspended in 3.0 ml of TM buffer containing 300 μ gm of lysozyme per ml and 15 μ gm of DNAase per ml. The suspension was kept at room temperature for 30 min at which time 0.5 ml of chloroform was added. This mixture was vigorously agitated for 15 min and then centrifuged to remove cell debris. The supernatant was combined with the original lysate supernate.

The combined supernate was made 2.0 *M* in ammonium sulfate and kept in the cold (4°C) for 3 hr. The phage which precipitates was removed by centrifugation at 10,000 rpm for 10 min. The phage was then suspended in 2.0 ml of TM buffer and dialyzed against TM buffer for 5 hr. After dialysis, the phage was layered on a 3–20% linear sucrose gradient containing 10^{-4} *M* MgCl₂ and centrifuged for 4 hr at 25,000 rpm at 5°C in an SW 25.1 rotor of the Spinco Model L. At the end of this time, the bottom of the swinging bucket tube was pierced and 1.0 ml fractions were collected and analyzed for radioactivity and plaque forming ability. The peak regions were collected and dialyzed against 3 changes of 0.01 *M* phosphate buffer. The phage preparation after dialysis was analyzed for optical density at 260 $m\mu$, plaque forming units and radioactivity.

(e) *Details of a conservation experiment:* *E. coli* K-10 cells were grown to a density of 5×10^8 cells per ml in 200 ml of SC medium at 37°C. They were washed twice with SC medium minus

glucose and finally resuspended in 10 ml of SC medium minus glucose. The N¹⁵P³² labeled MS ϕ 2 phage was then added to the cell suspension and the mixture was maintained at 37°C for 30 min without aeration.

After 30 min of adsorption 90 ml of pre-warmed complete SC medium was gently added to the phage-cell mixture. This is considered zero time of infection. After 1 min aeration by shaking was instituted. At 10 min the culture was chilled by swirling in an ice bath and then centrifuged at 6,000 rpm for 5 min. The sedimented cells were then washed two times with cold, glucose-free, SC medium containing 0.01 *M* versene. The wash medium was kept for analysis of removed radioactive phage. The infected cells were finally resuspended in pre-warmed complete SC medium. At this time, aliquots for infectious centers were taken. The culture was again shaken until 30 min had elapsed at which time bentonite was added to a final concentration of 5 mg per ml. The culture was then shaken for 30 more min. At this time the culture was again chilled. The suspension was centrifuged at 12,000 rpm for 15 min to separate the unlysed cells from the lysate. The cell pellet was analyzed for its radioactive content. The supernatant fraction was analyzed for radioactivity and was made 0.01 *M* in versene to chelate the Ca⁺⁺ present which otherwise formed an insoluble precipitate upon the addition of EtOH. Then 10 ml of 2.0 *M* potassium acetate and 220 ml of 100% ethanol was added to precipitate the nucleic acids in the supernate. The precipitate was collected by centrifugation at 10,000 rpm for 10 min and resuspended in 3 ml of TM buffer. To this solution was added 3.0 ml of water-saturated phenol and the mixture was vigorously agitated at room temperature for 15 min. The phenol and aqueous layers were separated by centrifugation at 6,000 rpm for 5 min. The aqueous layer was removed and extracted twice with 3 volumes of ether (anhydrous). The ether was removed by blowing nitrogen through the solution. Finally, the solution was made 0.2 *M* in potassium acetate and 2 volumes of 100% ethanol was added to precipitate the nucleic acid. The precipitate was resuspended in 2.0 ml of TM buffer and used for density gradient centrifugation in Cs₂SO₄. Final recovery of purified RNA from the lysate was between 60–70%.

Results.—(a) *Identification of parental strands in a population of RNA molecules:* An answer to the question of conservation requires the identification of the injected strand in a mixture of progeny and cellular RNA components. Obviously, the parental RNA would be present at levels precluding their identification as optically observable components. Consequently, two identifying isotopic labels were used, N¹⁵ to provide a unique position in a density gradient, and P³² to permit detection of the original strands.

Preliminary reconstruction experiments were carried out to see how readily N¹⁵-P³²-labeled phage RNA could be identified in the presence of its unlabeled counterpart and ribosomal RNA. A difficulty was encountered due to a tendency of phage RNA to aggregate with ribosomal RNA, if the latter is present in excess. This complication was avoided by preparing P³²-viral RNA of high specific activity, permitting the use of small aliquots, and adding unlabeled carrier viral RNA in excess to displace the labeled RNA from any existent complex with ribosomal RNA.

The separation of tritiated ribosomal RNA from unlabeled virus RNA in a cesium sulfate gradient is shown in Figure 1A. In this case the amount of ribosomal RNA added is small and its position is located by its H³ label. Figure 1B demonstrates that N¹⁵-P³²-labeled virus RNA can be easily distinguished from unlabeled viral RNA. The amount of N¹⁵-P³²-RNA added was deliberately kept low in the reconstruction experiment in order to conform to the conditions expected in an actual conservation experiment. Evidently, aggregation between the N¹⁵-P³² and the unlabeled RNA does not occur. It is clear from Figure 1 that density gradient centrifugation permits a ready identification of each type of RNA in a mixture, providing radioactive labeling is used in addition as an aid to location.

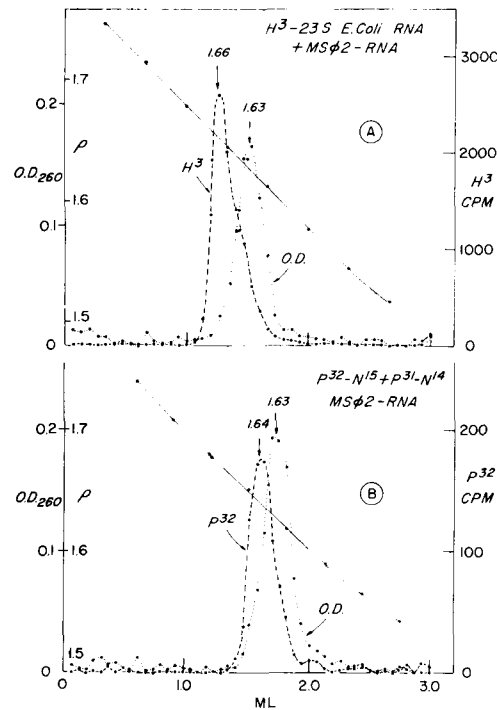


FIG. 1.—Fractionation of RNA by cesium sulfate density gradient centrifugation: (A) The cesium sulfate solution contained *E. coli* 23S RNA- H^3 (14 μ gm, 13,000 cpm) and MS ϕ 2 RNA- $N^{15}P^{31}$ (50 μ gm) in a final volume of 3.0 ml with a density of 1.612. The sample was centrifuged for 72 hr at 33,000 rpm at 25°C in the SW39 rotor of the Spinco Model L ultra-centrifuge. At the end, fractions of 0.061 ml were collected from the bottom of the tubes, diluted and analyzed for optical density at 260 $m\mu$ and radioactivity as described in *Methods*. The straight line gives the density gradient. (B) The cesium sulfate solution contained MS ϕ 2 RNA- $N^{15}P^{32}$ (0.05 μ gm, 1200 cpm) and MS ϕ 2 RNA- $N^{15}P^{31}$ (50 μ gm) in a final volume of 3.0 ml with a density of 1.612. Other details as in (A).

(b) *Evidence for complete conservation of the parental strands:* To obviate the complications mentioned in the introduction, and permit a definitive decision, the following precautions were introduced into the conservation experiment: (1) Low multiplicities of infection (0.05 or less) were used to avoid the possibility of having active and inactive strands in the same cell. (2) Unattached or reversibly attached particles were eliminated by washing the cells prior to lysis. (3) To insure restriction of the examination to infected complexes actively producing phage components, analysis was confined to the RNA released after lysis.

Since the virus particles are labeled with P^{32} , the various steps can be readily monitored. It was found that two centrifugal washings with buffered versene effectively removed all unadsorbed or reversibly attached virus particles. The counts which remain with the cells after the washing permit an upper limit calculation of the actual multiplicity of infection. To satisfy the third criterion, incubation had to be continued until lysis occurred and unlysed cells removed prior to isolation of the RNA. This required protection of the released RNA from nucleolytic degradation in the crude lysate. Using labeled phage RNA in reconstructed systems, it was found that the addition of bentonite¹⁵ permitted full recovery.

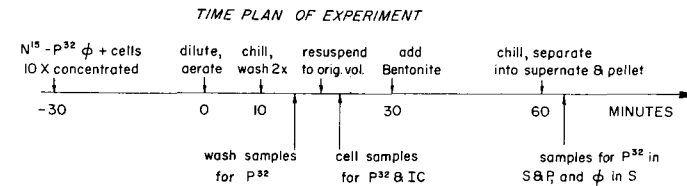


FIG. 2.—Outline for the conservation experiment: The times of additions, operations and removal of samples are indicated. The symbols employed denote the following: IC, infectious centers; S, lysate supernatant; P, lysate pellet; ϕ , phage count. See other details in *Methods*, section e.

As a result of these and other preliminary investigations a procedure for such experiments was finally evolved which yielded consistent results with satisfactory regularity. The time plan of such experiments is diagrammed in Figure 2 and a typical protocol detailed in *Methods* (section e).

Depending on the initial ratio of phage to bacteria, between 70–90% of the particles were lost in the wash fluids. The inputs of virus particles were accordingly adjusted to yield the finally desired multiplicities of infection. Table 1 summarizes a number of experiments carried out at different multiplicities. Recorded is the radioactive material which remains with the cells and is ultimately recovered as acid precipitable material after lysis has occurred. It will be noted that the per cent recovery of the fixed P^{32} -RNA is excellent, ranging from 95–98%. Further, between $1/3$ and $1/6$ is released into the supernatant fraction, the remainder being found in cells which have not lysed by 60 min. In addition, there is reasonably good agreement between the P^{32} -phage equivalents found in the lysate and the number of infectious centers assayed immediately after the washing and subsequent resuspension. The yield of active virus particles found per lysed cell in such experiments ranged from 400–900. This is somewhat lower than the 1,000–2,000 found in the same medium with cultures allowed to complete the lytic cycle undisturbed.

We now turn our attention to the P^{32} -labeled RNA found in the supernatant fractions of the lysates formed after 60 min. It may first be noted that in all cases it was found that this RNA is as sensitive to RNAase as added control RNA, suggesting that it is all free. Under the same conditions labeled RNA contained within intact virus particles is resistant to degradation. Of immediate interest is the question of whether the P^{32} is still in association with N^{15} . The RNA of the supernatant

TABLE 1
DISTRIBUTION OF P^{32} IN CONSERVATION EXPERIMENTS

Each experiment involved 100 ml of starting culture. In all cases the numbers represent total found. Conversion of counts to phage equivalents was done on the basis measuring the O.D. at 260 (1.10) and the cpm per ml (5.1×10^7) of the purified N^{15} - P^{32} -virus particles. On the basis of comparison of plaque formers in freshly purified phage with O.D. one finds that one O.D. is equivalent to ca. 1.7×10^{10} particles.¹³ Using these numbers yields a value of 2.7×10^{-6} cpm/particle. The multiplicity of infection was calculated by dividing the total counts irreversibly fixed in the washed cells in phage equivalents by the total cell number.

Experiment	A		B		C		D	
	Counts	ϕ -equivalents	Counts	ϕ -equivalents	Counts	ϕ -equivalents	Counts	ϕ -equivalents
Washed cells	10,300	3.7×10^9	23,400	8.4×10^9	47,600	1.7×10^{10}	93,400	3.4×10^{10}
Lysate pellet	8,310	3.0×10^9	19,350	7.0×10^9	35,530	1.3×10^{10}	70,700	2.5×10^{10}
Lysate supernate	1,400	0.5×10^9	4,700	1.7×10^9	9,700	3.6×10^9	19,200	7.0×10^9
Per cent recovery	94	—	98	—	95	—	96	—
Infectious centers	—	1.1×10^9	—	2.0×10^9	—	3.6×10^9	—	8.6×10^9
Cell number	—	8.7×10^{10}	—	9.5×10^{10}	—	8.5×10^{10}	—	9.0×10^{10}
Multiplicity of infection	—	0.042	—	0.084	—	0.2	—	0.38

fraction was purified according to the procedure detailed in *Methods*. Recovery of the final product ranged between 60–70%. It was then mixed with carrier P^{32} - N^{14} -virus RNA and banded in Cs_2SO_4 . A typical outcome is given in Figure 3. It is clear that virtually all of the P^{32} is still found in a strand possessing the density characteristic of the N^{15} -labeled viral RNA. Little, if any, of the P^{32} has found its way either into the ribosomal RNA density region or into that which corresponds to progeny viral RNA. The band width of the P^{32} -peak indicates that no breakdown leading to size dispersity has occurred. This was further confirmed by chromatography on columns of methylated albumin.¹⁸ In a number of repetitive experiments involving multiplicities of infection ranging from 0.04 to 0.09, identical results were obtained.

Discussion.—In the experiments described, the RNA examined is obtained from the lysate which contains active particles corresponding to an average burst of about 600 per lysed cell. This would seem to insure that the radioactive strands have been derived from cells actively synthesizing the necessary viral RNA and protein components. At the effective multiplicity employed (about 0.05), no more than 5 per cent of the infected cells would be expected to contain more than one viral equivalent. Since virtually all the RNA is accounted for, and purification yields 60–70% of the starting material, it would seem safe to conclude that a fair sample of actively participating RNA has been subjected to analysis.

The fact that the P^{32} bands in the density gradient at a location characteristic of the N^{15} -RNA, and that no P^{32} is found at densities corresponding to either newly formed viral or ribosomal RNA, argues for complete conservation. We conclude that the original strands of an RNA virus can be recovered intact at the end of a complete lytic cycle.

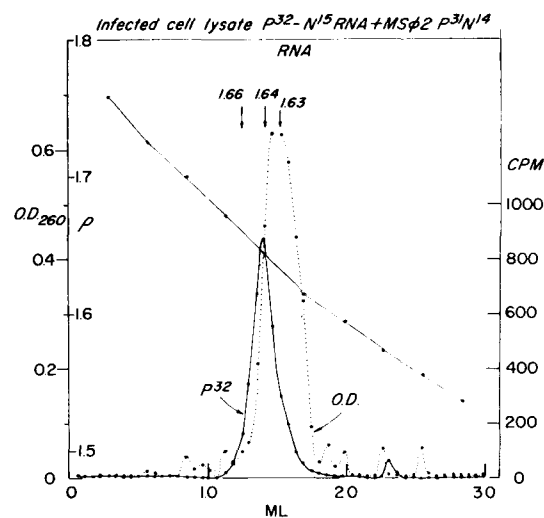


FIG. 3.—Identification of components in lysate RNA by density gradient centrifugation: The cesium sulfate solution contained the phenol purified lysate RNA (5,280 cpm and a total optical density of 0.5 at 260 $m\mu$) and 200 μ gm of marker $MS\phi 2$ RNA- $N^{14}P^{31}$ in a total volume of 3.0 ml at a density of 1.612. Other details as in legend for Figure 1 (A) and in section *e* of *Methods*.

It may be noted, in passing, that examination of the RNA in cells which have failed to lyse reveals that, in at least a portion, breakdown of the injected strand has occurred.¹⁶ This further emphasizes the necessity of focusing attention on those cells which can produce virus particles in any attempts at understanding the ultimate fate of the incoming viral RNA.

The fact that all the parental RNA found in the lysates is RNAase sensitive suggests that the input strands are excluded from the final maturation process. This result is consistent with the finding of Davis and Sinsheimer¹⁷ who, on direct examination of the mature virus yield, failed to detect original parental strands.

While the experiments summarized answer the question posed, they raise a number of other issues for experimental resolution. As pointed out in the introduction, there are good reasons for presuming that the incoming strand must serve as a structural program for a new protein. While the possibility of new RNA synthesis was not completely eliminated, the recent studies^{20, 21} on *in vitro* synthesis of viral proteins on addition of homologous viral RNA strongly suggest the same conclusion. Further, we have shown in results to be detailed elsewhere¹⁸ that the injected strand does indeed behave like a message. It is found in association with 80S ribosomes from the very onset and remains there throughout the course of the infection. The present experiments demonstrate, nevertheless, that they are conserved. However, indigenous genetic messages of these cells appear to be unstable and in continual turnover.^{9, 10, 19} We are faced with the problem of finding the mechanism which permits destruction of one message and not another. Whatever the outcome, it is clear that instability is not a necessary property of all RNA molecules which serve as translatable messages for protein synthesis.

The problems of replication and transcription of RNA genomes are especially intriguing since they can still provide us with an interesting deviation from the expected. In particular, it should be noted that DNA genomes *must* transcribe their information into complementary RNA copies for use in protein synthesis. On the other hand, we have seen that RNA genomes are already translatable messages. Consequently, complementary transcription is not only unnecessary but is indeed likely to result in the formation of a nonsense strand, useless for protein synthesis. Thus, if complementary copying occurs it would be employed only for replicative purposes. However, replication can, in principle, occur via identical copies by readily designable mechanisms. The possibility must, therefore, be entertained that we may yet find that the RNA viruses have completely bypassed the use of complementarity.

Summary.—In an attempt to get an answer to the question of conservation, the RNA of mature virus particles was doubly labeled by growth in the presence of N^{15} and P^{32} . The RNA recovered after completion of lysis was banded in gradients of Cs_2SO_4 . The two isotopes were recovered in the same RNA strands, corresponding in density to the N^{15} -RNA originally injected. The experiments were carried out under conditions which avoided the ambiguity which would be generated by the presence of nonparticipating strands or inactive virus particles.

The data are consistent with the conclusion that the parental strands of an RNA virus are completely conserved during all the replications and translations required to produce a full yield of mature virus particles. Since it is very likely that the incoming viral RNA must serve as a genetic message, the results indicate that in-

stability is not a mandatory attribute of RNA molecules which serve as programs for protein synthesis.

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