SPECIFICITY IN TRANSCRIPTION OF THE REOVIRUS GENOME*

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Reovirus contains double-stranded ribonucleic acid with a molecular weight of approximately 10^7 daltons.[1,2] This RNA exists in the virion as a continuous linear structure, but it is extraordinarily susceptible to breakage.[3-5] When isolated by any of the commonly used extraction techniques, the major portion of viral RNA appears as fragments with lengths of 0.35 μ, 0.6 μ, and 1.1 μ, corresponding to molecular weights of 0.8×10^6, 1.6×10^6, and 2.4×10^6 daltons respectively.[3] In previous work we have shown that the three fragments do not arise through random breaks; rather, there are specific weak spots in the RNA chain where the breaks occur during isolation.[6]

Further, the virus-specific single-stranded ribonucleic acid (ssRNA) formed in infected cells, and shown previously to be messenger RNA (mRNA),[7,8] was separated by sucrose gradient sedimentation into three clearly defined fractions whose sedimentation rates coincided with those found for the three double-stranded (ds) RNA fragments after denaturation.[6] For each of the three classes of dsRNA a similar length of mRNA could thus be isolated, suggesting that each mRNA might be copied from the corresponding segment of dsRNA in the infected cell. This proposition is shown to be correct in the present paper. Each class of ssRNA hybridizes exclusively with its corresponding length of denatured dsRNA and is, therefore, transcribed uniquely from it in the infected cell.

Methods.—Reovirus was propagated in suspension cultures of L cells.[9] In such cultures two species of virus-induced RNA commence to form at approximately five hours after infection and continue to be synthesized for a further ten hours or so.[8-10] One RNA is double-stranded and is viral progeny RNA. Its extraction and separation into three fractions by chromatography on columns of methylated bovine albumin-kieselguhr (MAK) have been described.[6] These fractions are designated dsRNA-1, dsRNA-2, and dsRNA-3, in order of elution from the column, and they have molecular weights of 0.8×10^6, 1.4×10^6, and 2.4×10^6 daltons, respectively. The other RNA formed in infected cells is single-stranded.[9] Its separation into three fractions by sucrose gradient sedimentation has been described.[6] Molecular weights of the three classes are 0.4×10^6, 0.7×10^6, and 1.2×10^6 daltons, and they are designated ssRNA-1, ssRNA-2, and ssRNA-3.[6]

Preparation of virus-specific RNA: Cellular RNA synthesis in infected cultures was suppressed by addition of 0.5 μg per ml of actinomycin D at the time of infection.[9] Double-stranded RNA was labeled with uridine-H^3 (0.2 μc per ml) or uridine-C^{14} (0.02 μc per ml) added four hours after infection. After a further 16 hours, the dsRNA was extracted with phenol and purified on a MAK column using a steep NaCl gradient that eluted all three dsRNA fragments in a single peak.[9] The eluate was concentrated by dialysis against Carbowax and was then dialyzed against 0.01 M STE buffer (0.01 M NaCl; 0.01 M Tris-HCl, pH 7.4; 0.001 M ethylenediaminetetracetate (EDTA))." This preparation contained dsRNA-1, dsRNA-2, and dsRNA-3, and will be referred to as dsRNA.

To prepare ssRNA, cycloheximide (20 μg per ml) was added to a culture eight hours after infection along with an additional 2 μg per ml of actinomycin D. Further synthesis of dsRNA was thus prevented, but ssRNA continued to form at an unabated rate.[10]   Uridine-H[3] (2 μc per ml) was added at 8.5 hours and the ssRNA was extracted with phenol 11.5 hours after infection and separated into its three constituent fractions.[6]

Conditions for annealing and hybridization:[6]   Labeled dsRNA, 40 μg in 0.1 ml of 0.01 *M* STE buffer, was denatured by addition of 1 ml of dimethyl sulfoxide (DMSO) and incubation at 37°C for 30 minutes.   After denaturation, 500 μg of yeast RNA was added as carrier and then 4 vol of ethanol.   The resulting precipitate was centrifuged, washed once with ethanol to remove DMSO, and redissolved in 0.4 ml of 0.3 *M* STE buffer (0.3 *M* NaCl; 0.01 *M* Tris-HCl, pH 7.4; 0.001 *M* EDTA).   Reannealing of the denatured RNA was effected by heating this mixture at 72.5°C for eight hours and then adding 2 vol of ethanol to precipitate the RNA. When ssRNA was to be hybridized, a known amount of it was added to the dsRNA before denaturation.   For electrophoresis, such reannealed RNA was dissolved in 0.05 ml of 0.01 *M* STE buffer containing 5 mM EDTA heated to 80°C for five minutes to break up aggregates that may have formed through the ethanol precipitation, and rapidly chilled.   A drop of 60 per cent sucrose containing a trace of bromphenol blue to act as a dye marker was added, and the mixture was layered over a column of acrylamide gel.

Acrylamide gel electrophoresis:[11]   Acrylamide solution was prepared by dissolving 8.55 gm of acrylamide and 0.45 gm of NN'-methylene bisacrylamide in 100 ml of 0.486 *M* tris-HCl buffer at pH 8.4.   A second solution, prepared just prior to use, contained 0.1 gm ammonium persulphate, 0.05 ml N,N,N',N'tetramethylethylenediamine in 50 ml of water.   Equal volumes of the two solutions were mixed and to the mixture was added an equal volume of hot 2 per cent agarose (Bausch & Lomb) solution in 0.243 *M* Tris-HCl (pH 8.4).   The resulting mixture, containing a final concentration of 2.25 per cent acrylamide and one per cent agarose, was quickly poured into glass tubes (75 mm × 5 mm inside diameter) which had been treated with dimethyldichlorosilane to minimize zone deformation at the walls of the tubes during electrophoresis.   A snugly fitting plug of parafilm had been previously inserted into the bottom of the glass tube to a distance of 10 mm.   When the agarose had partially solidified, the hemisphere of gel protruding from the end of the glass tube was cut off with a razor blade, a drop of buffer was placed on the flat gel surface, and this end of the tube was sealed with a piece of dialysis tubing secured with a rubber band.   The tube was inverted and the parafilm plug carefully removed. Water was then layered over the resulting gel surface and the tubes were maintained vertically in a beaker containing a small amount of buffer for 18 hours.   Before use, the water on top of the gel was removed with filter paper, the sample to be analyzed was added, and the remainder of the tube was filled with the reservoir buffer (0.03 *M* diethylbarbituric acid; 0.0033 *M* Tris, pH 8.5).   Electrophoresis was carried out at 4 ma per tube for 100–120 minutes in a standard form of apparatus.[11] After the run, the dialysis membrane was removed from the bottom of the tube, the gel was extruded under gentle pressure and sliced with a razor blade into pieces approximately 0.9 mm thick.

Two different methods were used to assay radioactivity in the gel slices.   In the

first method the individual slices were placed on glass fiber filters (2.4 cm diameter) in glass scintillation-counting vials. One milliliter of 3 N ammonium hydroxide was added and the capped vials were kept at 37° for 18 hours. The contents were then evaporated to dryness at 110°, 2 ml of scintillation fluid (4 gm of 2,5-diphenyl-oxazole (PPO) and 0.32 gm of p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene) was added and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

The second method permitted subsequent recovery of the RNA from the gel. Gel slices were placed in vials containing 8 ml of toluene scintillation fluid and 2 ml of methanol and the contents were shaken at intervals during the next four hours. After being assayed for radioactivity, the RNA was extracted from the gel slices by incubating them at 4° for 18 hours in one ml of 0.3 M STE buffer containing 5 mM · EDTA. No more than 50 per cent of the RNA was recovered from the gel in this way.

Results.—In outline, the object was to determine whether each piece of ssRNA would hybridize exclusively with one of the fragments of dsRNA. A test became feasible when it was found that the various classes of ssRNA and dsRNA could be separated from each other by electrophoresis on acrylamide gels, and that after re-annealing a mixture of ssRNA and denatured dsRNA, all the ssRNA was hybridized and migrated with the three dsRNA fractions on electrophoresis. Each ssRNA component in turn could then be annealed with a mixture of the three denatured dsRNA fragments and the product analyzed by gel electrophoresis to find whether the ssRNA had hybridized with one or more of them.

Analysis of dsRNA and ssRNA by electrophoresis through acrylamide gels: Figure 1 shows the pattern obtained when dsRNA was subjected to electrophoresis. In this experiment, H³-labeled dsRNA was mixed before analysis with C¹⁴-labeled
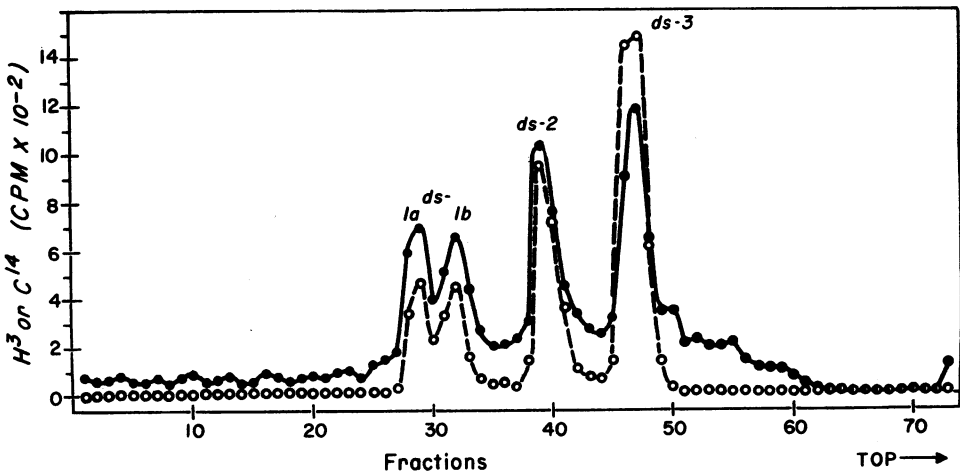

Fig. 1.—Electrophoretic analysis of dsRNA and of denatured and reannealed dsRNA. H³-labeled dsRNA, 1.3 μg, 15,000 cpm (O---O), was mixed with C¹⁴-labeled dsRNA, 15 μg, 20,000 cpm (●—●), that had been denatured and reannealed as described in *Methods*. The mixture was analyzed by electrophoresis on an acrylamide gel column. ds-1a and ds-1b are components of dsRNA-1; ds-2 is dsRNA-2; ds-3 is dsRNA-3.

dsRNA which had previously been denatured and then reannealed. The three classes of dsRNA-H[3] separated cleanly and migrated in the same order as they were eluted from MAK columns.[6] In fact, dsRNA-1, which previously appeared as a single peak by sucrose gradient sedimentation or by MAK column chromatography, was separated into two components. Moreover, the peaks of C[14] and H[3] were in identical positions, showing that the reannealing of denatured dsRNA-C[14] had restored the original double-stranded structure.

When a mixture of H[3]-labeled ssRNA and C[14]-labeled dsRNA was analyzed by electrophoresis, the ssRNA separated into three fractions that moved ahead of the dsRNA components (Fig. 2a). In other experiments, not described here in detail, we have found that the three components of denatured dsRNA migrate in the same positions as does ssRNA upon electrophoresis. Part of the material whose analysis is shown in Figure 2a was mixed with DMSO to denature the dsRNA and then heated to reanneal the separated strands. Analysis of the product gave the pattern shown in Figure 2b. The only significant peaks of H-[3]labeled material ran with the three dsRNA fractions. This result indicated that the ssRNA had hybridized with complementary strands from dsRNA during the reannealing process and, moreover, that the resulting products were similar in electrophoretic properties to the three fractions of dsRNA prior to denaturation.

Hybridization of individual ssRNA classes with dsRNA: The previous results made feasible the critical experiment to determine whether each class of ssRNA would hybridize uniquely with a single class of dsRNA. Single-stranded RNA
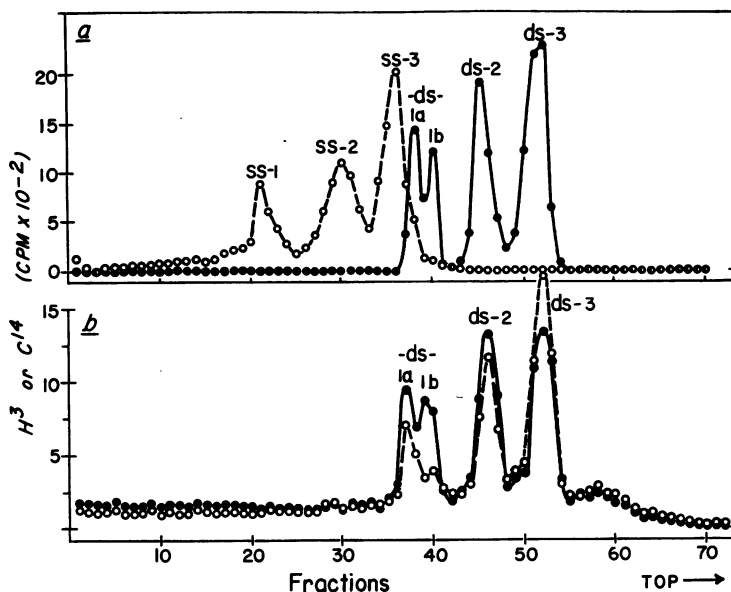

FIG. 2.—Electrophoretic analysis of a mixture of ssRNA and dsRNA before and after hybridization. H[3]-labeled ssRNA, approximately 1 μg, 50,000 cpm (O—O), was mixed with C[14]-labeled dsRNA, 40 μg, 67,000 cpm (●—●), in 0.1 ml of 0.01 M STE buffer. (a) Part of the mixture was analyzed immediately; (b) an equal part was denatured and reannealed as described in *Methods* and then analyzed. ss-1 is ssRNA-1; ss-2 is ssRNA-2; ss-3 is ssRNA-3.

labeled with H³ was separated into its three constituent components. Each component was mixed with a specimen of C¹⁴-labeled dsRNA. These mixtures were treated with DMSO to separate the double strands, heated to reanneal them, and then analyzed by gel electrophoresis. The results (Fig. 3) show that ssRNA-1 hybridized exclusively with dsRNA-1, ssRNA-2 with ds-RNA-2, and ssRNA-3 with dsRNA-3. It should be mentioned that the concentration of ssRNA in the hybridization tests was relatively so much lower than that of dsRNA that the excluded C¹⁴-labeled strand of dsRNA would not be seen in the patterns of Figure 3.

Hybridization between the single- and double-stranded RNA was clearly highly specific but the extent of homology in each case had still to be found. The test for this purpose depended on the fact that reovirus RNA is resistant to digestion by RNase, becomes sensitive on denaturation, and again becomes resistant to RNase upon reannealing. In fact, this response to RNase has already been used to de-
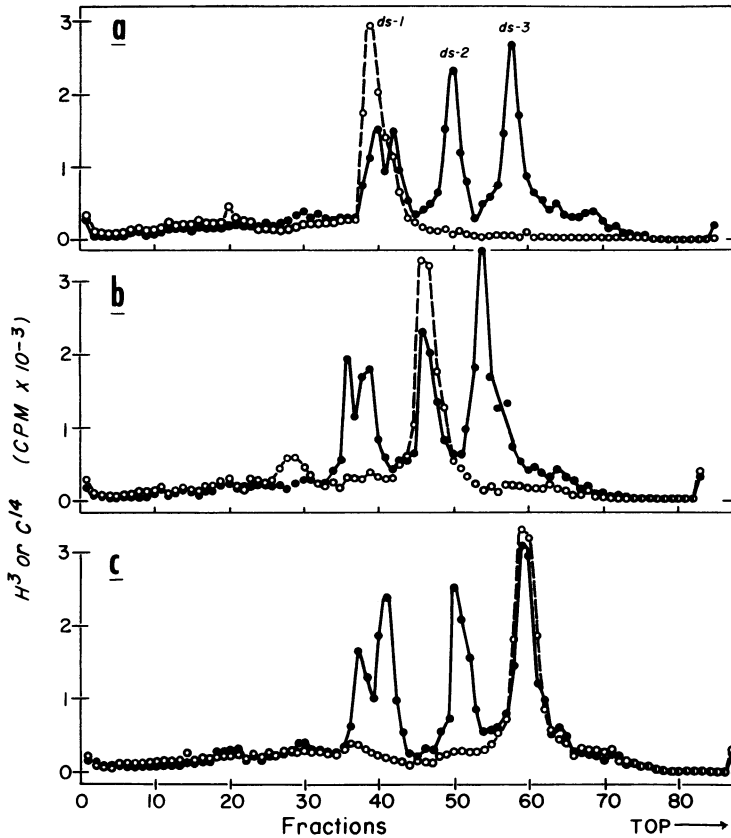

FIG. 3.—Hybridization of the three species of ssRNA with dsRNA. Each of H³-labeled ssRNA-1, ssRNA-2, and ssRNA-3, approximately 1 μg, 60,000 cpm, and 0.1 ml of 0.01 *M* STE buffer (O---O), was mixed separately with a sample of C¹⁴-labeled dsRNA, 40 μg, 67,000 cpm, in 0.2 ml of 0.01 *M* STE buffer (●—●). These mixtures were denatured, reannealed and analyzed by electrophoresis. (*a*) ssRNA-1-H³ with dsRNA-C¹⁴; (*b*) ssRNA-2-H³ with dsRNA-C¹⁴; (*c*) ssRNA-3-H³ with dsRNA-C¹⁴.

termine the kinetics of reannealing of denatured dsRNA.[6]   In the present instance, the three hybrid fractions shown in Figure 3 were isolated and tested for their sensitivity to RNase.[6]   Approximately 90 per cent of both the C^{14}-and H^3-labeled RNA resisted digestion by 5 μgm per ml of RNase for 30 minutes at 37°C.   Thus the homology was almost perfect between each class of ssRNA and a complementary strand from its matching length of dsRNA.

Discussion.— Two conclusions may be drawn from these results.   First, there are no significant regions of homology between the three fragments of dsRNA since each class of ssRNA hybridized with only one class of dsRNA.   This finding strengthens our previous contention[6] that these dsRNA segments arise through breakage at specific weak points in the viral genome rather than by random scissions. Second, the three pieces of isolated dsRNA represent the sections of viral genome from which the three classes of mRNA are copied in infected cells.   Full stops for transcription of the various polycistronic messages coincide with the physically weak regions in the genome.   It is clearly of interest to determine the chemical structure of these regions.

From the H^3-profile of Figure 1, the relative amounts of the three classes of ds-RNA may be determined on the assumption that uracil is randomly distributed in viral RNA.   This computation has been done for twelve such analyses on four different preparations of dsRNA.   The total amount of H^3 in each profile was normalized to 100 cpm.   Mean values, with their standard errors, of 24.4 ± 0.8, 30.1 ± 0.24, and 45.5 ± 2.1 cpm were obtained for the relative amounts of dsRNA-1, dsRNA-2, and dsRNA-3 respectively, giving an average molar ratio of 1.6/1.1/1 for dsRNA-1/dsRNA-2/dsRNA-3.   Tentatively, we thus suggest that the viral genome may contain three sections of dsRNA-1, two of dsRNA-2, and two of dsRNA-3, a total of seven pieces, which would provide a molecular weight of 10^7 daltons in agreement with the value derived from chemical analysis of purified virus.[1]

In fact, some separation of dsRNA-1 fragments was achieved as shown in Figure 1 (ds-1a, ds-1b).   Although resolution of these double-stranded pieces was poor, it is of considerable interest that relatively little ssRNA hybridized with the fraction dsRNA-1b (Figs. 2b and 3a).   Perhaps this section of the genome is copied at a slower rate than the others, or during a different interval in the infectious cycle than we have studied here.   We shall examine the problem of the control of transcription in this system in a later paper.

At the present time, the reovirus system seems unique in that sections of the viral genome and their corresponding mRNA's can be readily isolated.   While the problem of separating all seven pieces of dsRNA and the seven equivalent mRNA's from each other is still to be solved, it now seems a feasible objective to allocate specific functions to the various segments of viral genome through the analysis of a series of conditional lethal mutants of reovirus.

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