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Separation of Ten Reovirus Genome Segments by Polyacrylamide Gel Electrophoresis

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Double-stranded ribonucleic acid (RNA) extracted from purified reoviruses of all three serotypes and from type 3 virus-infected cells was analyzed by polyacrylamide gel electrophoresis. It was calculated that each RNA includes 10 segments: 3 large, 3 intermediate, and 4 small fragments corresponding to molecular weights of about 2.5, 1.4, and 0.8 × 10⁶ daltons, respectively, or a total of 15 × 10⁶ daltons.

Reoviruses contain an amount of double-stranded ribonucleic acid (RNA) equivalent to a minimum molecular weight of 10×10^6 daltons (8). Although a duplex structure of this size would have a contour length of 4 to 5 μm, RNA extracted from purified reovirus includes some molecules which are 7 to 8 μm in length (6, 7, 9). This finding suggests that the reovirus genome is intact within the virion and has a molecular weight close to 15×10^6 daltons. Recently, an estimate of 17 to 22×10^6 daltons has been obtained from microscopy measurements (21).

There is also evidence that weak points exist along the length of the reovirus genome. During isolation from the virus particles by a variety of procedures, reovirus RNA fragments into a reproducible mixture of short segments (7, 10, 17); these fall into three size groups designated L, M, and S which correspond to molecular weights of about 2.5, 1.4, and 0.8×10^6 daltons, respectively (3, 22). Fragmentation of the genome apparently results from breakage at specific sites rather than from random scissions, since there is no significant base sequence homology between RNA fragments of different size (1, 22, 23). It is clear from its estimated minimum molecular weight of 10^7 daltons that the viral genome is not simply composed of one of each of the L, M, and S segments. In the present report, the double-stranded RNA segments obtained from all three reovirus serotypes have been resolved further by polyacrylamide gel electrophoresis. The electrophoretic patterns are in agreement with a molecular weight of 15×10^6 daltons for the reovirus genome.

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MATERIALS AND METHODS

Carrier-free ^{32}P-orthophosphate and uridine-2-^{14}C (20 μc/μmole) were purchased from International Nuclear & Chemical Corp., City of Industry, Calif., and Calbiochem, Los Angeles, Calif., respectively. Reovirus serotypes 1 (Lang), 2 (D-5 Jones), and 3 (Abney) were obtained from the American Type Culture collection. Mouse L-929 and HeLa S3-1 cells were grown in Eagle's medium containing 5% fetal bovine serum.

The procedures for the preparation of stock virus pools, infection of cells, virus purification, phenol extraction of RNA from virions and infected cells, and the separation of double-stranded and single-stranded RNA by methylated albumin-kieselguhr (MAK) column chromatography have been described previously (19). Polyacrylamide gel electrophoresis of viral RNA in 10-cm gels at 20 C and 4 ma per gel was performed by the method of Loening (12). Gels containing radioactive RNA were frozen and cut into 1.3-mm slices (5). The slices were dissolved in 0.1 ml of hydrogen peroxide by heating for 20 hr at 60 C; 1 ml of NCS reagent (Nuclear Chicago Corp., Des Plaines, Ill.) and 10 ml of liquifluor-toluene were added for counting (14). Stained gels were fixed for 15 min in 1 M acetic acid, stained for 1 hr in 0.4 M acetate buffer (pH 5) containing 0.2% methylene blue, and decolorized in water (15).

RESULTS

Analysis of double-stranded RNA extracted from purified type 3 reovirus. Double-stranded RNA isolated from reovirus type 3 was separated into eight distinct bands by electrophoresis in 2.5% polyacrylamide gels (Fig. 1). The slowly migrating single band, 2.5 cm from the origin, corresponds to the class of large RNA fragments (L) which have a contour length of 1.1 μm and a molecular weight of 2.5×10^6 daltons calculated from sedimentation measurements (3, 6, 17, 22, 23). A

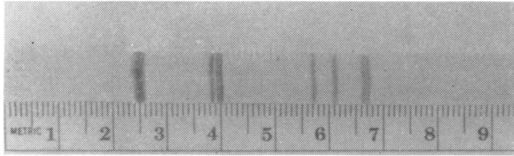

FIG. 1. *Electrophoretic pattern of type 3 reovirus double-stranded RNA; 2.5% polyacrylamide gel; 13 μg of RNA; migration time, 7 hr.*

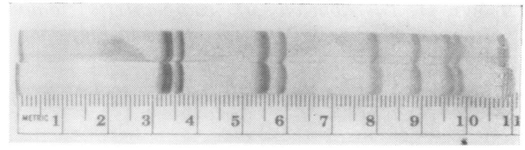

FIG. 2. *Patterns of RNA from purified type 3 virus grown in L cells (upper) or HeLa cells (lower); 5% gel, 11 and 15 μg of RNA, 17 hr.*

second class of fragments migrated 4 cm and included three distinct bands. They represent the RNA segments which are intermediate in size (M), with a length of 0.6 μm and a molecular weight of about 1.4×10^6 daltons. The class of shortest segments (S), previously described to be about 0.35 μm in length and of molecular weight 0.8×10^6 daltons, consisted of four separate RNA bands which migrated 5.7, 6.1, 6.6, and 6.7 cm from the origin.

An increase in the resolution of the large RNA molecules was obtained by raising the gel concentration to 5.0% and the duration of electrophoresis from 7 to 17 hr. Under these conditions, the slowly migrating RNA was resolved into three L bands (Fig. 2). Identical electrophoretic patterns were obtained for double-stranded RNA extracted from type 3 reovirus grown in L cells or HeLa cells indicating that the size distribution of the genome fragments is not influenced by the host cell.

When ^{32}P-labeled type 3 reovirus RNA was analyzed by electrophoresis in 5% gels, three size-categories were again evident (Fig. 3). However, the resolution obtained by slicing and counting the gels was less than that obtained with stained gels. Of the three L segments (fractions 25 to 29), two moved as a single peak (I) and the third was only partially resolved (I'). A similar separation into two peaks (II and III) was observed for the M class of RNA molecules (fractions 38 to 44). Two of the four S segments were well separated (peaks IV and V), but the two fast components moved as a single peak (VI). An estimate of the expected distribution of radioactivity among the various peaks was made on the basis of the number of different RNA fragments resolved in the stained gels and their molecular weights as determined from sedimentation analysis and electron microscopic measurements. As shown in Table 1, good agreement was found between the observed and estimated values.

Analysis of double-stranded RNA from virus-infected cells. It was of interest to determine whether the double-stranded RNA fragments obtained from purified virions corresponded in

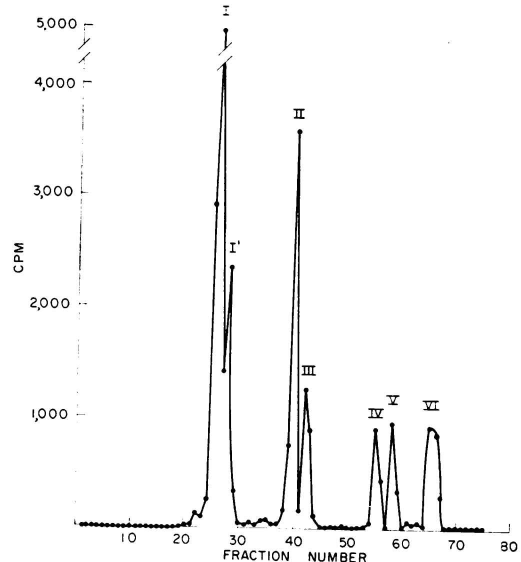

FIG. 3. *Electrophorogram of ^{32}P-labeled type 3 RNA; 5% gel, 17 hr. Migration from left (cathode) to right (anode) in this and subsequent figures.*

number and size to those synthesized in reovirus-infected tissue culture cells. Reovirus replication in L cells is unaffected by concentrations of actinomycin sufficient to inhibit cellular RNA synthesis by more than 90% (11, 18). Under these circumstances, the synthesis of reovirus RNA can be detected at about 6 hr after infection and continues for 8 hr or longer, i.e., throughout most of the infectious cycle. L cells in suspension culture were infected with type 3 virus, and 0.3 μg of actinomycin per ml was added 1 hr later. The culture was divided and the resulting replicates were incubated with uridine-2-^{14}C for 2 hr beginning at 6, 8, and 10 hr after infection. At the end of the labeling period, total RNA was extracted with phenol and precipitated with ethyl alcohol. The double-stranded RNA was separated by MAK column chromatography, reprecipitated with ethyl alcohol, and analyzed by electrophoresis in 5% polyacrylamide gels. As shown in Fig. 4, the migration patterns of RNA formed 8 to 10 and

TABLE 1. *Distribution of radioactivity among reovirus type 3 RNA fragments in Fig. 3*

Peak	Observed counts/min	Estimated molecular wt equivalents[a]	Estimated counts/min[b]
I + I′	12,460	7.5 × 10^6	12,247
II	4,783	2.8 × 10^6	4,572
III	2,242	1.4 × 10^6	2,286
IV	1,439	0.8 × 10^6	1,306
V	1,313	0.8 × 10^6	1,306
VI	2,128	1.6 × 10^6	2,612
Total	24,365	14.9 × 10^6	

[a] Values based on the patterns shown in Fig. 1 and 2 and the average molecular weights for L, M, and S RNA molecules of 2.5 × 10^6, 1.4 × 10^6, and 0.8 × 10^6 daltons, respectively (3, 6, 7, 17, 22).

[b] Total counts/min divided by total estimated molecular weight = 1,633 counts per min per 10^6 daltons. Estimated counts/min equals 1,633 multiplied by estimated molecular weight equivalents in each peak.

in units of 10^6 daltons 10 to 12 hr after infection were almost identical to that of RNA extracted from purified virions (Fig. 3). The RNA made early in infection (6 to 8 hr) was also similar except that there was no separation of the L segments (fractions 22 to 26, peaks I and I′). The distribution of radioactivity among the different bands in Fig. 4 is given in Table 2. As for virion RNA (Table 1), the values in each case are in agreement with the presence of three large, three intermediate, and four short lengths of RNA. The finding that the radioactivity distribution was the same for the RNA synthesized during all three time-intervals suggests that the rates of formation of the different genome segments do not change relative to each other during infection. However, these results would also be obtained if the cultures were asynchronously infected.

Comparison of the genomes of reovirus types 1, 2, and 3. Double-stranded RNA molecules isolated from purified virions of all 3 serotypes were examined. A distinctive staining pattern was found for each type and the small differences among serotypes were reproducible (Fig. 5). The L and M RNA segments of types 1 and 3 were each resolved into three bands. In the case of type 2 RNA, there were only two L and two M bands, but the faster band in each class was more intensely stained, indicating that it included two unresolved segments of nearly identical molecular weight. Each virus type included four segments of low molecular weight, although the two most rapidly migrating molecules were poorly resolved. This was particularly evident with the RNA from type 1 virus.

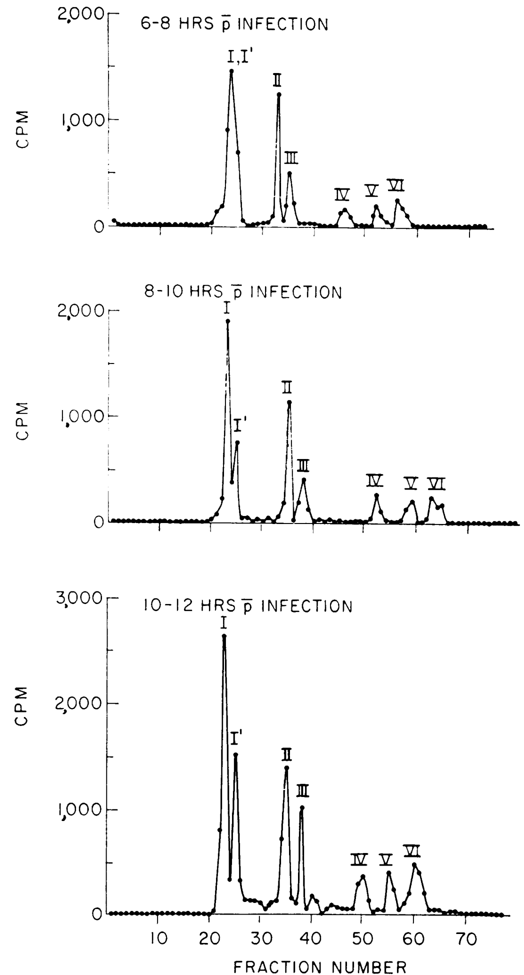

FIG. 4. *Separation of double-stranded RNA segments synthesized at different times after infection of L cells with type 3 reovirus.*

The relative amounts of RNA in the different bands was determined for virus types 1 and 2 in the same manner as type 3. Double-stranded RNA was isolated from purified virus which had replicated in the presence of ^{32}P-orthophosphate, and the radioactive RNA molecules were analyzed in 5% gels. As observed for type 3 RNA, the staining procedure resulted in greater resolution of bands than was obtained by counting gel slices (Fig. 6). However, the count profiles always agreed with the staining patterns. The three L segments of types 1 and 2 RNA were not separated. The three intermediate type 1 fragments were resolved, but only two intermediate peaks of type 2 RNA were present. As in the stained gel patterns of type 2 RNA, the faster of the two intermediate peaks contained more material. On

TABLE 2. *Distribution of radioactivity in Fig. 4*

Peak	Estimated molecular wt equivalents	Counts/min at 6–8 hr		Counts/min at 8–10 hr		Counts/min at 10–12 hr	
		Estimated[a]	Observed[b]	Estimated	Observed[c]	Estimated	Observed[d]
I + I′	7.5×10^6	3,480	3,488	3,530	3,434	6,250	5,756
II	2.8×10^6	1,300	1,399	1,320	1,431	2,340	2,402
III	1.4×10^6	650	724	658	729	1,170	1,180
IV	0.8×10^6	372	379	376	431	669	890
V	0.8×10^6	372	348	376	355	669	749
VI	1.6×10^6	744	588	751	630	1,340	1,485
Total	14.9×10^6		6,926		7,010		12,462

[a] Values for type 3 RNA calculated as described for Table 1.
[b] Counts per min per 10^6 daltons was 465.
[c] Counts per min per 10^6 daltons was 470.
[d] Counts per min per 10^6 was 835.

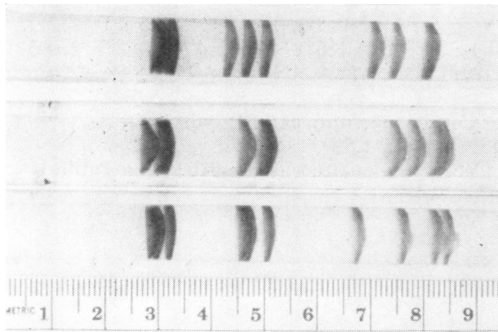

FIG. 5. *Comparison of double-stranded RNA molecules extracted from purified reovirus serotypes 1 (upper), 2 (middle), and 3 (lower).*

the basis of the count distribution (Table 3), it is clear that the faster peak included two M segments of similar molecular weight and electrophoretic mobility. The short segments of types 1 and 2 RNA separated into 3 peaks; the fast peak also included two unresolved fragments.

As reported previously for single-stranded RNA (4), the mobility of double-stranded RNA in polyacrylamide gels is proportional to molecular weight (Fig. 7). It is also apparent from their separation in polyacrylamide gels that the reovirus RNA segments within each class have slightly different molecular weights. An estimate of their sizes was made by plotting the average distances which the three classes of type 3 segments migrated against their average molecular weights, determined by sedimentation analysis (Fig. 7). The size of individual fragments was then estimated from their electrophoretic mobilities. The values obtained in units of 10^6 daltons were 2.5, 2.4, and 2.3 for the three L segments: 1.6 for Ma and Mb and 1.4 for Mc (Fig. 7); and 0.92, 0.76, 0.64, and 0.61 for the four S segments or a total of 14.7×10^6 daltons.

DISCUSSION

Separation of double-stranded reovirus RNA into two M, two L, and three S fragments by gel electrophoresis has been reported previously (3). By varying the experimental conditions and using a gel staining procedure, 10 different segments, including three L, three M, and four S pieces, have been resolved in RNA preparations from type 3 reovirus. Similar patterns were obtained with RNA from types 1 and 2, indicating that the molecular weight of the reovirus genome is about 15×10^6 daltons for each serotype. In addition to the double-stranded genome, reoviruses contain an amount of single-stranded, adenine-rich RNA equivalent to one-fourth of the molecular weight of the duplex or about 3.7×10^6 daltons (2, 3, 20). The combined molecular weight of the viral RNA is 18.4×10^6 daltons.

The three reovirus serotypes are morphologically similar (13) and share a common complement-fixing antigen (16). In addition, virions of each type contain three major and four minor protein components of comparable electrophoretic mobility (P. Loh and A. Shatkin, Bacteriol. Proc., p. 170, 1968). These findings, together with the similarity of the RNA patterns, make it likely that the genomes of the different serotypes are related. It has been found that single-stranded, virus-specific RNA species from L cells infected with virus types 1 and 3 hybridize with genome segments of comparable length from purified virions of either serotype (S. Millward, Y. Watanabe, and A. F. Graham, Bacteriol. Proc., p. 172, 1968). By the same hybridization criteria, types 2 and 3 are much less related than 1 and 3 (Shatkin, *unpublished results*). Further studies of

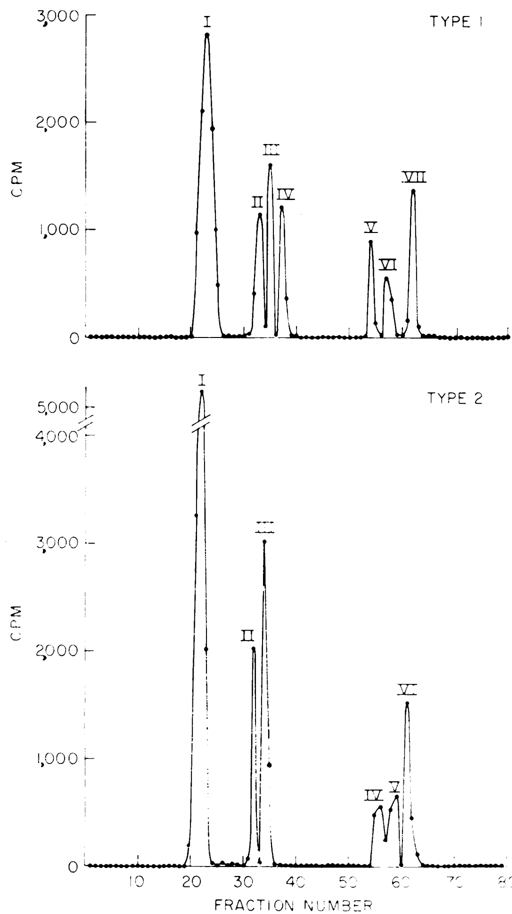

FIG. 6. *Electrophorograms of ^{32}P-labeled RNA from virus types 1 and 2.*

homologous regions among RNA molecules of the three serotypes will be facilitated by combining the gel staining procedure and autoradiographic techniques.

Although the genomes of most viruses are isolated as polycistronic nucleic acids, RNA extracted from reovirus includes fragments ranging in size from 2.5 to 0.6 $\times 10^6$ daltons. Each of the L segments contains enough nucleotides to code for several proteins, whereas the smallest S segments are probably monocistronic. It is of considerable interest to learn how the genetic information of reoviruses is distributed among the RNA pieces. One obvious approach is to test the separated fragments in an in vitro protein-synthesizing system. Although double-stranded reovirus RNA does not stimulate amino acid incorporation in *Escherichia coli* extracts, denatured viral RNA is active. However, the reaction products are smaller than the proteins present in purified virions (Shatkin, *unpublished results*). It remains to be determined whether this approach will be possible with an in vitro system from mammalian cells.

TABLE 3. *Distribution of radioactivity in Fig. 6*

Peak	Estimated molecular wt equivalents	Counts/min[a]	
		Estimated[b]	Observed
Type 1			
I	7.5×10^6	8,450	8,449
II	1.4×10^6	1,580	1,679
III	1.4×10^6	1,580	1,618
IV	1.4×10^6	1,580	1,564
V	0.8×10^6	904	1,027
VI	0.8×10^6	904	955
VII	1.6×10^6	1,808	1,543
Total	14.9×10^6		16,835
Type 2			
I	7.5×10^6	11,300	11,723
II	1.4×10^6	2,110	2,117
III	2.8×10^6	4,220	4,003
IV	0.8×10^6	1,205	1,278
V	0.8×10^6	1,205	1,212
VI	1.6×10^6	2,410	2,093
Total	14.9×10^6		22,426

[a] Counts per min per 10^6 daltons was: type 1, 1,130; type 2, 1,510.

[b] Values calculated as described for Table 1.

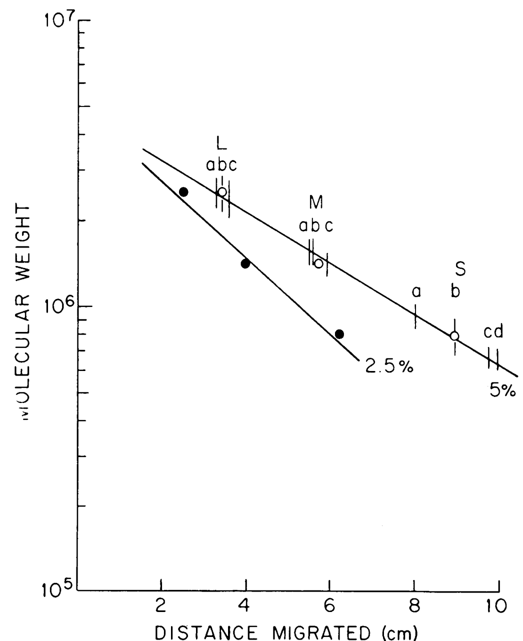

FIG. 7. *Relationship between electrophoretic mobility and molecular weight of type 3 double-stranded RNA segments in 2.5 and 5% gels.*

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