*STUDIES ON THE A-RICH RNA OF REOVIRUS**

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Recent evidence suggests that the RNA of reovirus, which is double-stranded,[1] possesses an unusual structure. Although it is sometimes possible, under very special circumstances, to liberate from virions nucleic acid strands sufficiently long to correspond to entire genomes,[2] lysis usually releases double-stranded RNA molecules of three distinct and reproducible sizes[3] with molecular weights of approximately 2.3×10^6, 1.3×10^6, and 8×10^5. These pieces are not random breakage products, since they serve as the intracellular templates for the transcription of messenger RNA molecules, which also fall into a characteristic trimodal size distribution.[4]

In addition to these double-stranded RNA segments, a class of single-stranded RNA molecules is also present within the virions. These are very rich in adenine (over 85 %) and constitute about 15–20 per cent of the total RNA present in reovirus.[3] We report here the results of some studies on the synthesis and properties of the A-rich RNA.

Methods.—Methods for the purification of reovirus, extraction of RNA from virions, and its analysis by means of sucrose density gradient centrifugation have been described before.[3, 4]

Electrophoresis of RNA in polyacrylamide gels: RNA samples were deproteinized by the method of Oda and Joklik,[5] precipitated with ethanol, and subjected to electrophoresis through 2.4, 10, or 15 per cent polyacrylamide gels, using the EDTA-phosphate buffer and fractionation techniques described previously.[3] Inclusion of EDTA in 10 or 15 per cent polyacrylamide gels interfered with polymerization; these gels were therefore subjected to electrophoresis in the EDTA-phosphate buffer system for two hours prior to the application of RNA samples.

Base composition: This was determined as described previously.[3]

Materials.—C^{14}-adenine (10 mc/mmole), H^3-adenine (6 c/mmole), and C^{14}-uridine (25 mc/mmole) were products of Schwarz BioResearch. P^{32}-orthophosphate was obtained from Squibb Laboratories, New York. T1 ribonuclease and pancreatic ribonuclease were purchased from the Worthington Biochemical Corporation. Ribonuclease T2 was a generous gift of Dr. Fuji Egami, Tokyo University, Japan. C^{14}-labeled and unlabeled poly A and unlabeled poly U were obtained from Miles Laboratories.

Results.—Measurement of A-rich RNA: A-rich RNA was detected orginally in the top fractions of sucrose density gradients charged with radioactively labeled reovirus lysed with SDS.[3] However, radioactivity in such fractions is a measure of the amount of A-rich RNA only in virion lysates and is inapplicable to cell extracts, since its sedimentation behavior is too similar to that of 4*S* transfer RNA. Attempts were made to develop an assay based upon the resistance of A-rich RNA to digestion by ribonuclease T2 after its hybridization with poly U. However, although hybridization is very efficient, as will be shown below, the method was not

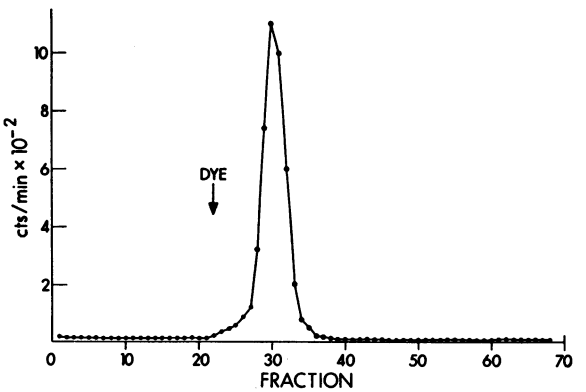

FIG. 1.—Polyacrylamide gel electropherogram of reovirus A-rich RNA. A-rich RNA was prepared from virus labeled with C^{14}-adenine (200 μc per 6 $\times$ 10^8 infected cells in 20 Blake bottles, total volume 1 liter) and was separated from viral double-stranded RNA by preliminary zone sedimentation in 15–30 % SDS-sucrose density gradients. A-rich RNA was recovered by precipitation with ethanol after addition of nonradioactive L cell RNA as carrier, as described before.[3] 100 μg of the resulting RNA mixture in 0.1 ml buffer (0.05 *M* NaCl, 0.02 *M* Tris, pH 7.5, 0.02 *M* EDTA) was mixed with 0.1 ml 30 % sucrose containing bromphenol blue as marker, and the mixture subjected to electrophoresis for 8 hr at 75 volts through a 20-cm 10 % polyacrylamide gel. Electrophoretic migration was from left to right.

satisfactory because other nonhybridizable RNA species in the preparations were also resistant to ribonuclease T2.

A sensitive assay procedure was then developed based on the finding that A-rich RNA displays a unique electrophoretic mobility in polyacrylamide gels. Figure 1 shows a typical profile. A-rich RNA migrates as a tight band in 10 (or 15) per cent gels, and gives no indication of resolving into components. It moves faster than 4*S* transfer RNA, and this supports the earlier conclusion that it is smaller.[3] In 10 per cent gels its *Rf* relative to 4*S* transfer RNA (which has the same mobility as bromphenol blue) is 1.37. Measurement of the amount of radioactivity in this band provides a sensitive assay of A-rich RNA. Thus, in one standard preparation, 1 mc of adenine-H^3 (6 c/mmole) was added to 6 $\times$ 10^8 L cells infected with reovirus (in 20 Blake bottles, total volume 1 liter). The A-rich RNA derived from the viral yield had a specific activity of about 1.6 $\times$ 10^4 cpm/μg under the counting conditions used. Amounts of A-rich RNA around 10 mμg can thus be readily detected, corresponding to approximately 3 $\times$ 10^9 virions. The sensitivity of the method can be further increased by more intensive labeling.

Synthesis of A-rich RNA: It is of considerable interest to know when A-rich RNA is synthesized during the viral multiplication cycle, and where it is synthesized. These questions could be answered once the assay procedure described above had been developed.

A culture of mouse L cells was divided into two equal parts, one of which was infected with reovirus, while the other was mock-infected. At various times thereafter samples of these two cultures were pulse-labeled for one hour with H^3-adenine,

disrupted by Dounce homogenization, and the cytoplasmic fractions centrifuged at 10,000 g for ten minutes. RNA was prepared from the pellet (P) and supernatant fractions (S) as described in the legend to Figure 2, and subjected to electrophoresis.

Panels $A(S)$ and $A(P)$ of Figure 2 show electropherograms of fractions S and P derived from uninfected cells pulse-labeled for one hour, 10 hours after mock infection. In fraction S, 4S transfer RNA forms a large peak, but there is no ma-

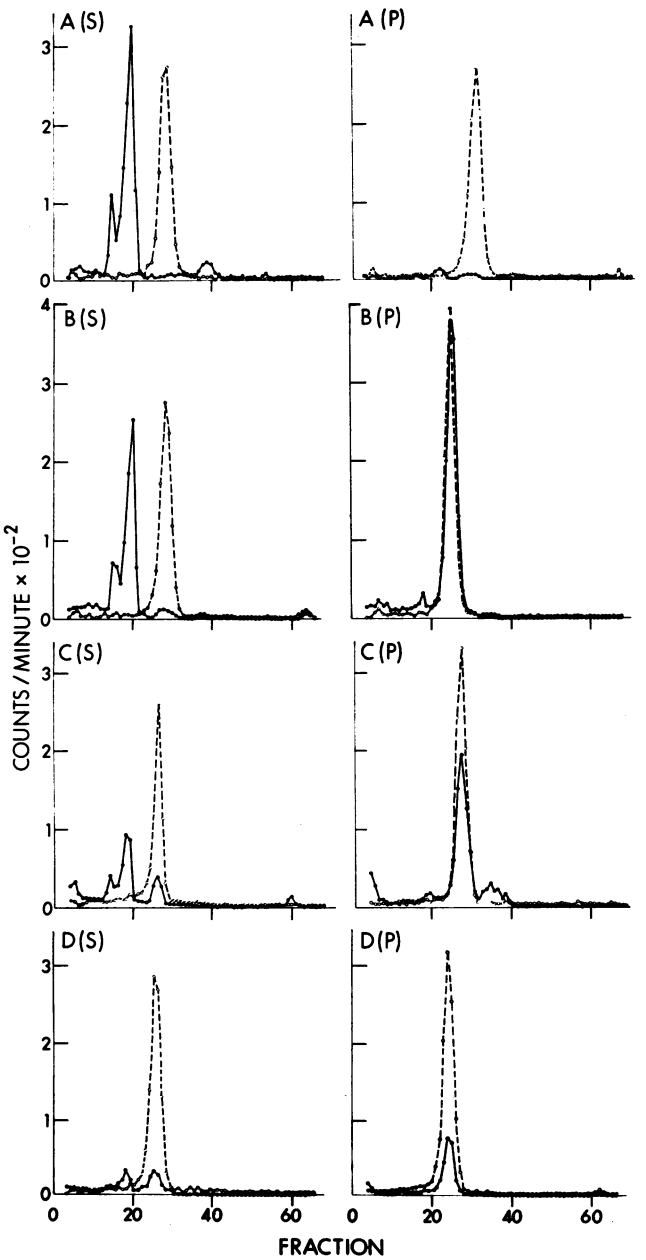

FIG. 2.—Effect of reovirus infection on the presence of A-rich RNA in L cell cytoplasm.

Samples of 5×10^7 L cells, infected or mock-infected, in 166 ml of medium containing 0.5 μg/ml actinomycin D, were pulse-labeled for 1 hr with 100 μc H^3-adenine.

Cytoplasmic fractions were prepared and separated into pellet (P) and supernatant (S) fractions by centrifuging at 10,000 $\times$ g for 10 min. RNA in these fractions was liberated by treatment with SDS, deproteinized by the method of Oda and Joklik,[5] precipitated with ethanol, and dissolved in electrophoresis buffer. The resulting solution (0.3 ml) was mixed with a marker containing C^{14}-adenine-labeled A-rich RNA prepared from purified virions, and the mixtures were subjected to electrophoresis on 10% polyacrylamide gels for 7 hr at 75 volts. Under these conditions reovirus genome and messenger RNA do not migrate beyond the first four fractions.

A, B, C, and D are described in the text.

—— H^3-labeled material derived from cytoplasmic fractions.

---- C^{14}-labeled A-rich RNA derived from virions.

terial corresponding to the A-rich RNA marker; in fraction *P* there is neither 4*S* transfer RNA nor A-rich RNA. Similar electropherograms from cell samples pulse-labeled for one hour at 12, 14, and 16 hours after mock infection likewise showed no A-rich RNA. We conclude that A-rich RNA is *not* synthesized in uninfected L cells.

The pairs of panels labeled *B*, *C*, and *D* show the corresponding electropherograms from infected cells pulse-labeled at 10, 14, and 16 hours after infection, respectively. During the period from 10 to 11 hours after infection there is active synthesis of A-rich RNA, practically all of which is deposited by centrifugation at 10,000 *g* for 10 minutes. During the 14- to 15-hour period the rate of synthesis of A-rich RNA is only about one half that during the 10- to 11-hour period; and although a small amount of this material is found in the supernatant, most is again deposited at 10,000 *g*. During the 16- to 17-hour period the rate of synthesis of A-rich RNA is lower still. It may be noted that the rate of incorporation of label into 4*S* transfer RNA follows a similar decline, as does the synthesis of reovirus genome RNA and reovirus messenger RNA.[4]

Other experiments showed that little, if any, A-rich RNA is synthesized before 8 hours after infection. The bulk is thus synthesized between 9 and 13 hours after infection. This time course coincides with that of reovirus genome RNA replication. Further, A-rich RNA is either synthesized in association with structures large enough to be deposited by centrifugation at 10,000 *g* for 10 minutes, or it becomes associated with such structures within a short time after its formation.

Presence of A-rich RNA in reovirus grown in different hosts: A-rich RNA was first detected in reovirus which had been allowed to multiply in mouse L fibroblasts.[3] In order to dispose of the possibility that synthesis and subsequent encapsidation of A-rich RNA are irrelevant epiphenomena in that specific system, reovirus which had been allowed to multiply in BHK 21 cells and in HeLa cells was examined for the presence of this RNA. A-rich RNA was present in all virus preparations, and is thus a constituent of reovirus irrespective of the nature of the host.

Base composition of A-rich RNA: We have reported previously the base composition of A-rich RNA isolated from sucrose density gradients to be 81 per cent A, 13 per cent U, 3 per cent C, and 3 per cent G.[3] However, such material may well be contaminated with small amounts of viral genome RNA broken down during extraction. This possibility is relevant to the question of whether A-rich RNA contains any G and C, which is of importance in relation to hypotheses concerning its mode of synthesis and function. The base composition of electrophoretically purified P^{32}-labeled A-rich RNA was therefore determined, and was found to be 87.8 per cent A, 10.5 per cent U, 1.4 per cent C, and 0.3 per cent G.

Since A-rich RNA is smaller than 4*S* transfer RNA, it contains fewer than 80 nucleotide residues, and a guanine content of 0.3 per cent represents less than one residue per molecule. If guanine were indeed a constituent of some molecules, these should be split by ribonuclease T1, and there should result a recognizable change in their electrophoretic behavior (provided guanine is not terminal). However, when electrophoretically purified A-rich RNA was exposed to a sample of ribonuclease T1 known to be active in digesting ribosomal RNA, its mobility was not detectably affected. The sensitivity of the test was such as to exclude the possibility of guanine being an internal constituent of more than one in 20 of A-rich

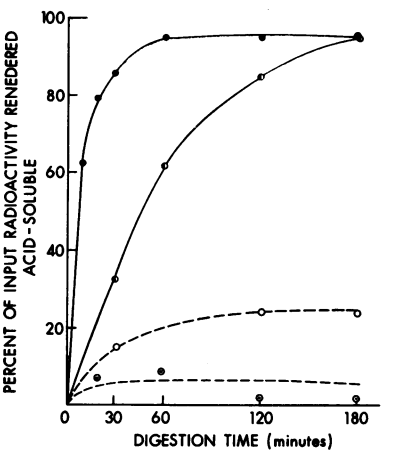

FIG. 3.—Hybridization of A-rich RNA with poly U. A-rich RNA labeled with H^3-adenine was prepared from radioactive virus and separated from virion double-stranded RNA by preliminary zone sedimentation as described above. Hybridization was carried out in 0.2 ml 6 × SSC pH 5.0, in sealed glass ampoules. Samples were heated for 14 hr at 65°; this was then followed by slow cooling to room temperature over a further period of 12 hr. Each sample was diluted to 2.0 ml with 6 × SSC and incubated at 37° with 0.04 units of T2 ribonuclease. The extent of digestion was followed by removing samples from the reaction tubes and measuring the amount of radioactivity rendered acid-soluble. ——●——, H^3-labeled reovirus A-rich RNA (3000 cpm), annealed alone; ——◐——, C^{14}-poly A (5000 cpm, 30 mμmoles), annealed alone; —○—, H^3-labeled reovirus A-rich RNA (3000 cpm), annealed with 150 mμmoles poly U; —⊙—, C^{14}-poly A (5000 cpm, 30 mμmoles), annealed with 150 mμmoles poly U.

RNA molecules. The most likely conclusion is that A-rich RNA does not contain guanine at all.

To examine the possibility that thymidine is in some way associated with A-rich RNA, reovirus was grown in L cells in the presence of C^{14}-thymidine. Purified virions were lysed with SDS, the lysate was centrifuged in sucrose density gradients, and the material from the top of the gradients then subjected to electrophoresis in polyacrylamide gels. No label could be detected in the A-rich RNA region. By contrast, A-rich RNA labeled with C^{14}-uridine formed a peak similar to that exhibited by A-rich RNA labeled with adenine.

Ability of A-rich RNA to hybridize with poly U: A-rich RNA hybridizes readily with poly U. Figure 3 shows the time course of digestion by ribonuclease T2 of A-rich RNA from reovirus and of authentic poly A, both before and after hybridization with poly U. Clearly both poly A and reovirus A-rich RNA hybridize with poly U and are thereby converted to a form in which they are largely resistant to ribonuclease T2.

Hybridization of A-rich RNA with double-stranded reovirus genome RNA: We have discussed in an earlier publication the possible significance of the A-rich RNA,[3] and in particular, the possibility that the segments of double-stranded RNA which together make up the reovirus genome may be joined by pieces of A-rich RNA. In order to explore this possibility further, attempts were carried out to hybridize A-rich RNA to double-stranded genome RNA. Preliminary experiments showed that whereas hybridization of A-rich RNA with poly U could be readily detected by the development of resistance to digestion with ribonuclease T2, this method was not sensitive enough to demonstrate hybridization with double-stranded RNA. This was not surprising, since it is conceivable that only a few nucleotides of A-rich RNA pair with short complementary single-stranded regions at the ends of double-stranded RNA molecules. The following is a much more sensitive assay. A-rich RNA, labeled with H^3-adenine, was hybridized with double-stranded reovirus genome RNA labeled with C^{14}-adenine. The mixture was then subjected to electrophoresis in polyacrylamide gels. Appropriate controls were carried out with A-rich RNA and double-stranded genome RNA alone, and with mixtures which had not been annealed. The electropherogram depicted in Figure 4 was that exhibited

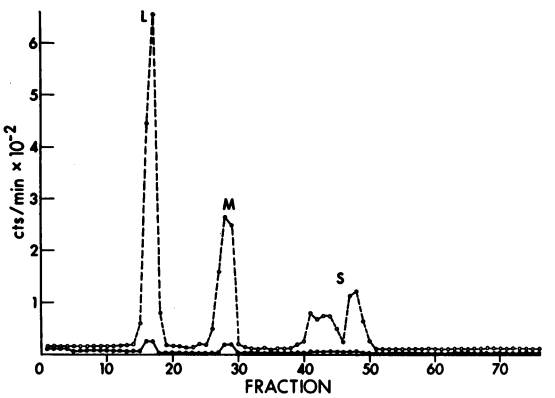

FIG. 4.—Attempts to hybridize reovirus A-rich RNA with reovirus double-stranded genome RNA. Reovirus double-stranded RNA labeled with C^{14}-adenine and A-rich RNA labeled with H^3-adenine were prepared from the respective virus preparations by zone sedimentation in 15–30 % SDS-sucrose density gradients. 7600 cpm C^{14}-double-stranded RNA (specific activity approximately 1.3×10^3 cpm/mμmole) was mixed with 8500 cpm H^3-A-rich RNA (specific activity 4×10^4 cpm/mμmole). The resulting mixture, in 0.1 ml 6 × SSC, pH 5, was heated in a sealed ampoule at 80° for 3 hr and then cooled slowly to room temperature over a period of 15 hr. The annealed mixture, diluted to 0.2 ml in electrophoresis buffer, was applied to a 20-cm 2.4 % polyacrylamide gel and subjected to electrophoresis at 75 volts for 10 hr. Broken line, C^{14} radioactivity (L, M, and S double-stranded reovirus genome RNA pieces); solid line, H^3 radioactivity (A-rich RNA).

by the mixture *after annealing*. The distribution of C^{14} label was exactly the same as that inhibited by genome RNA alone without annealing, and was very similar to that described earlier:[3] there were typical L, M, and S peaks of double-stranded RNA, the last already partially resolved. There was no evidence that any of these molecules had acquired an altered electrophoretic mobility as a result of annealing, as would be expected if two double-stranded RNA molecules had been linked by a molecule of A-rich RNA. Further, there was no H^3 label anywhere in the electropherogram; free A-rich RNA molecules migrate so much faster than double-stranded genome RNA molecules that they had moved out of the gel. The specific activity of the A-rich RNA used in this experiment was about 35 times that of the double-stranded genome RNA. The binding of one molecule of A-rich RNA to as few as one out of every five double-stranded genome RNA molecules could therefore have been detected. The conclusion from this experiment is that A-rich RNA does not link to pieces of double-stranded reovirus genome RNA *in vitro*.

Discussion.—The following is the extent of our knowledge concerning reovirus A-rich RNA. It is synthesised in mouse, hamster, and human cells infected with reovirus, but not in uninfected cells. It is synthesized at about the same time as reovirus double-stranded genome RNA, and is either synthesized in structures deposited by centrifugation at 10,000 *g* for ten minutes, or becomes associated with such structures very soon after it is formed.

A-rich RNA is single-stranded, as evidenced by the fact that it is readily digested by ribonuclease T2. It may be hybridized to poly U and is then resistant. It displays a remarkably homogeneous behavior upon electrophoresis in polyacrylamide gels: there is no sign that it can be resolved, even after prolonged electrophoresis in concentrated gels. Whether all molecules of A-rich RNA are identical remains to be determined.

The base composition of electrophoretically homogeneous A-rich RNA is 88 per cent A, 10.5 per cent U, and 1.5 per cent C. A very small amount of G (0.3%) is probably an impurity since ribonuclease T1 has no detectable effect on the electro-

phoretic behavior of A-rich RNA, and therefore does not hydrolyze it; G cannot be an internal constituent of more than 5 per cent of A-rich RNA molecules. Further, even if G is terminal, it cannot be present in all molecules, since A-rich RNA most probably contains no more than 60 nucleotides, and only 1 in every 300 bases is G. Whether C is a constituent remains to be determined. The amount found would correspond to one C per molecule.

The function of A-rich RNA remains a mystery. A sensitive hybridization and electrophoretic procedure gave no evidence of linkage to pieces of double-stranded reovirus genome RNA. The amount of A-rich RNA may often be 15–20 per cent of that of double-stranded genome RNA. Assuming that A-rich RNA contains 60 nucleotides, this corresponds to 50–100 molecules per virion, and this large number makes a linker function unlikely. It is of considerable interest in this connection that mammalian DNA has recently been found to contain a very large number of identical sequences which are probably no more than 400 nucleotides in length.[6] The function of this DNA is likewise a mystery.

It is not known how A-rich RNA is synthesized. Newly formed molecules of A-rich RNA detectable in cytoplasmic extracts of infected cells are exactly the same size, as judged by their rate of migration in polyacrylamide gels, as the molecules present within virions. A-rich RNA is thus apparently not a randomly synthesized polymer, with molecules of the right size happening to become encapsidated; on the contrary, it would appear that *only* molecules of a certain size are formed. It is therefore of great interest to determine the nucleotide distribution in A-rich RNA, for such knowledge may well suggest possible modes of synthesis: for instance, it may indicate whether it is formed on a template.

Summary.—Infection of three different cell strains of widely differing origin with reovirus elicits the formation of single-stranded RNA which is encapsidated in virions together with double-stranded genome RNA segments. Electrophoresis in polyacrylamide gels provides a convenient and highly specific means of detection and quantitation of this RNA. Its base composition is A 88, U 10.5, C 1.5, and G probably 0. The molecules are small, probably of the order of 60 nucleotides in length, and are remarkable uniform in electrophoretic behavior. Each virion contains about 50–100 of these molecules. Hybridization to poly U is readily demonstrated, since the A-rich RNA is thereby rendered resistant to ribonuclease T2; however, hybridization or linking to double-stranded reovirus genome RNA segments cannot be demonstrated.

Abbreviations used: A, adenine; U, uracil; C, cytosine; G, guanine; poly A, polyadenylic acid; poly U, polyuridylic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; SSC, 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.

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