Assay for Secondary Structure in Ribonucleic Acid

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A chromatographic procedure is described which can discriminate among single-stranded ribonucleic acid (RNA) molecules in solution on the basis of the extent of their secondary structure. The assay is effected through chromatography at different temperatures with columns of cellulose CF-11. When Sindbis virus RNA is chromatographed in this system, the ratio of the amounts of RNA eluting in the single-stranded peak to those eluting in the double-stranded peak increases at higher temperatures, presumably a measure of the relative amounts of Sindbis virus RNA secondary structure at different temperatures. With this assay, Sindbis virus RNA, phage f2 RNA, and polyuridylyate have been found to have different amounts of secondary structure.

Much effort has been expended in the determination of the structure of single-stranded ribonucleic acid (RNA) (see 3 and 10 for reviews), and it is generally conceded that in solution these molecules have hairpin-like segments composed of double-stranded hydrogen-bonded regions lying in an antiparallel configuration. One of the difficulties encountered in studies of RNA secondary structure is the lack of a convenient assay for its presence. This paper reports the development of a technique, using columns of cellulose CF-11, which distinguishes the presence or absence of secondary structure in RNA molecules.

Columns of cellulose CF-11 have been used by Franklin (5) to separate single-stranded R17 phage RNA from the multi-stranded replicative intermediate and further by Robertson et al. (9) to separate all types of single-stranded RNA from multi-stranded RNA molecules which contain double helical regions. These separations were effected in two single-step elutions from a column of cellulose CF-11, the first in buffer with 15% ethanol and the second in buffer alone. Single-stranded RNA elutes primarily in the 15% ethanol step.

The present assay grew out of the observation that single-stranded Sindbis virus RNA does not elute entirely in the 15% ethanol step, as expected because of its single-stranded nature, but rather elutes partially in the 0% ethanol step, as if it were in part double helical. Because Sindbis virus RNA is known to possess a high degree of secondary structure in solution (11), we attempted to explain its aberrant chromatographic behavior as being a function of this structure.

MATERIALS AND METHODS

Growth and purification of Sindbis virus. The stocks of Sindbis virus used were grown on chick embryo cells (CEC). Twenty-four hours before infection, 10^8 CEC from a primary culture were plated on 840-cm² Bellco glass roller bottles (Bellco Co., Vineland, N.J.). These 2° CEC were grown at 10^6 cells/ml of NCI medium (Gibco, Grand Island, N.Y.) supplemented with 6% calf serum (CS) (Microbiological Associates, Bethesda Md.). They were infected with Sindbis virus [HR strain (2)] at a multiplicity of 5 plaque-forming units (PFU) per cell in a volume of 10^7 cell/ml of adsorption medium (NCI plus 3% CS) for 20 min at 37 C. After this adsorption period, the volume of the medium was brought to 10^6 cell/ml with NCI plus 3% CS. To prevent interferon-induced viral interference, either 0.5 μ g of actinomycin D per ml was added at this time or partially purified virus (interferon free; see below) was used for infection. At 3 hr after infection, ³H-uridine (Schwarz/Mann, Orangeburg, N.Y.) was added to a final concentration of 1 μ Ci/ml. After 18 hr of virus growth, the supernatant liquid was removed, and the virus yield was determined by plaquing. The yield was routinely 500 to 1,000 PFU per cell. Plaquing was done on CEC monolayers in 60-mm petri dishes by the agar overlay method.

The Sindbis virus was purified by the method of Strauss, Burge, and Darnell (12), although individual steps were varied. After the ammonium sulfate precipitation step, the virus was subjected to two runs of sucrose-deuterium density gradient banding followed by a velocity sucrose gradient sedimentation step. (The Genesolv D step was omitted.) The final purified virus had a specific activity of about 10^{10} PFU/optical density unit at 260 nm (OD_{260}), and the recovery was 15 to 25% of the initial viral PFU.

Extraction and characterization of RNA in Sindbis virus. Viral RNA was extracted from the Sindbis stocks by bringing them to 0.1% in sodium dodecyl sulfate. This RNA sedimented as a single peak on a

sucrose gradient, as can be seen in Fig. 1. Furthermore, Sindbis virus RNA chromatographed as a single species with a molecular weight of approximately 4×10^6 in formaldehyde during electrophoresis in an acrylamide gel (7). Since formaldehyde reacts with RNA to destroy secondary structure (1), this last observation is taken to mean that the 45S Sindbis virus RNA molecule routinely isolated from pure virus particles is composed of one major species of single-stranded RNA. The absence of even short double helical regions is supported by the sensitivity of Sindbis RNA to pancreatic ribonuclease (11; D. L.

FIG. 1. Sedimentation of ^3H -uridine-labeled Sindbis virus RNA in a sucrose gradient. Sedimentation is from right to left. A 1.0-ml amount of Sindbis virus RNA was layered onto a 30-ml preformed 5 to 20% sucrose gradient in TSE made 0.1% in sodium dodecyl sulfate. The RNA was centrifuged in a Spinco 25.1 head at 19,000 rev/min at 15 C for 10 hr. After centrifugation 20 samples were taken by pumping sucrose from the bottom of the tube. The amount of radioactivity in 0.1 ml of each of these fractions was determined by diluting this fraction in 3 ml of cold 5% trichloroacetic acid and collecting the precipitate on Whatman GF/A glass fiber filters.

Engelhardt, unpublished data) under conditions that do not permit digestion of double helical RNA (4).

Source and characterization of phage f2 RNA and poly U. Phage f2 was grown and purified and the RNA was extracted as described previously (14). Phage f2 RNA, like Sindbis virus RNA, is entirely sensitive to pancreatic ribonuclease digestion (Engelhardt, unpublished data) under conditions that do not permit digestion of double helical RNA. It also sediments as a single peak in sucrose gradients (8). Based on these data we conclude that f2 RNA is a single molecule of single-stranded RNA. The polyuridylylate (poly U) in this work was obtained from Schwarz/Mann (lot W-2024). The average molecular weight was 7.6×10^6 by their determination. This poly U sedimented as a uniform peak with a relative *S* value of 3.5 in a buffer (TSE) composed of tris(hydroxymethyl)aminomethane, saline, and ethylenediaminetetraacetic acid.

Cellulose CF-11 chromatography. The preparation and use of cellulose CF-11 columns has been described elsewhere (4, 5, 9). In this work the running buffer TSE was used at pH 7.2 rather than 6.8. Runs at 37 and 4 C were performed in a thermostatted warm room and cold room, respectively, to insure proper equilibrium to the buffers. The radioactive RNA was precipitated by addition of cold (>20 C) trichloroacetic acid, collected on Whatman glass fiber filters, and assayed in a liquid scintillation counter. The OD was determined in a Beckman DU spectrophotometer. In all cases, the recovery of input material from the column was monitored and the individual recoveries are reported in the figure legends.

RESULTS

Elution profile of Sindbis virus RNA from cellulose CF-11 columns. Because it is single stranded, RNA from purified Sindbis virus would be expected to elute from a cellulose CF-11 column in the 15% ethanol step. As can be seen in Fig. 2, about 40% eluted in the 0% peak, which would be expected if it were partially double helical.

To determine whether this chromatographic procedure had separated two classes of Sindbis virus RNA, one containing a double-stranded region, we rechromatographed both peaks (Fig. 3). The RNA from the 0% peak (Fig. 3, solid line) again had an elution profile of about 60% in the 15% ethanol step and about 40% in the 0% ethanol step. The material from the second 0% ethanol step was chromatographed a third time (figure not shown) with the same results, i.e., about 60% eluted in the 15% ethanol step and about 40% eluted in the 0% ethanol step. When the RNA in the original 15% ethanol step (Fig. 2) was rechromatographed (Fig. 3, dashed line), about 70% eluted in the 15% ethanol step and about 30% eluted in the 0% ethanol step.

From this set of chromatographic runs we concluded that Sindbis virus RNA can exist in two states, one which elutes in the 15% ethanol

FIG. 2. Chromatography of ^3H -uridine-labeled Sindbis virus RNA on a column of cellulose CF-11. The column (12 mm by 9 cm) was eluted by gravity flow. The 35% ethanol buffer was equilibrated under 15 lb. of pressure to eliminate the bubbling in the column caused when air comes out of solution. The determination of radioactivity was made on a single 0.5-ml sample fraction as described in Materials and Methods. Background has been subtracted, and the arrows marked EtOH 35%, 15%, and 0%, respectively, indicate the fraction at which the elution buffer containing that amount of ethanol was initiated. Samples of approximately 2 ml were collected, and the reported radioactivity should be multiplied by four to obtain a number representing the total recovery. For this particular column, 2.1×10^4 counts/min were recovered from a column onto which 2.4×10^4 counts/min had been added.

step and one which elutes in the 0% ethanol step. These two states must be in equilibrium in solution, since RNA from both the 15% ethanol elution step and the 0% elution step rechromatograph with essentially identical elution profiles.

Effect of temperature on elution profiles of Sindbis virus RNA. If the equilibrium between the two states described above is a function of the formation or breakage of weak chemical linkages, then the position of this equilibrium should be affected by temperature changes. Figure 4 shows the results of three CF-11 column runs at three different temperatures and illustrates the temperature dependence of the reaction. At 37 C (solid line), about 75% of the input RNA eluted in the 15% ethanol step, whereas the rest eluted in the 0% ethanol step. At 23 C (dashed line), the

FIG. 3. Rechromatography on cellulose CF-11 of ^3H -uridine Sindbis virus RNA. The solid profile was obtained by adding 1 ml of the material from fraction 20 (the 0% ethanol peak tube) of Fig. 2 to a column (12 mm by 9 cm). Each 2-ml fraction was counted by adding an equal volume of 10% cold trichloroacetic acid. Thus the total counts in this figure represent the percentage of recovery in these columns (no counts eluted in the 35% elution step). In this column approximately 1,300 counts/min were added and 900 counts/min were recovered. The dashed profile was obtained by adding 1 ml of the material from fraction 13 (the 15% ethanol peak tube) of Fig. 2 column; approximately 700 counts/min added and 800 counts/min were recovered. In these plots background counts have been subtracted.

proportions were about 60% to 40%. At 4 C (dashed and dotted line), the proportions were reversed, with about 35% of the input RNA eluting in the 15% ethanol step and about 65% eluting in the 0% ethanol step.

These data are replotted in Fig. 5, in which the dashed line represents the percentage of the input RNA that elutes in the 15% ethanol step and the solid line represents the percentage of the input RNA that elutes in the 0% ethanol step as a function of temperature. From this figure it can be seen that at about 15 C 50% of the Sindbis virus RNA will be in each elution step. Further, if the dashed curve is extrapolated, 100% of the RNA will elute in the 15% ethanol step at about 55 C. This temperature is close to the published melting temperature (65 C) of Sindbis virus RNA (11).

Effect of temperature on elution profiles of phage f2 RNA. RNA from the bacteriophage f2 also displays a temperature-dependent alteration of chromatographic behavior on cellulose CF-11

FIG. 4. The effect of temperature on the elution characteristics of ^3H -labeled Sindbis virus RNA. This figure depicts three separate runs at 34 C (solid line), at 23 C (dashed line), and at 4 C (dashed and dotted line) through columns (12 mm by 9 cm). Fractions of 2 ml were taken. The solid line represents the radioactivity obtained by precipitation of 0.5 ml of each fraction as described in Materials and Methods. The counts recovered were 2,400 or a total of 9,600 counts/min. To the column were added 8,500 counts of ^3H -label per min. The dashed profile is a replot of Fig. 2. The dot and dash profile was run similarly to the solid line profile. Total recovery was 5,700 counts/min of an input 8,500 counts/min.

FIG. 5. A plot of the percent of ^3H -uridine-labeled Sindbis virus RNA in either the 15% ethanol elution step (dashed line) or the 0% ethanol elution step (solid line) at various temperatures.

columns, as illustrated in Fig. 6. The solid line in this figure depicts an elution profile obtained at 23 C, at which about 95% of the RNA elutes in the 15% ethanol step. [Similar results were reported when the f2 RNA was chromatographed at room temperature (see reference 4).] The dashed line in this figure depicts the elution profile obtained when phage f2 RNA was chromatographed at 4 C, at which about 25% of the input RNA eluted in the 15% ethanol step and about 75% in the 0% ethanol step. Thus phage f2 RNA behaves on cellulose CF-11 chromatography like Sindbis virus RNA in that both seem to have two forms, one of which chromatographs in the 15% ethanol step and the other in the 0% ethanol step. However, at room temperature, phage f2 RNA exists solely in a form which elutes in the 15% ethanol step, whereas Sindbis virus RNA exists in both forms at room temperature.

When *Escherichia coli* 16S ribosomal RNA and HeLa S3 total ribosomal RNA species were chromatographed on cellulose CF-11 columns at 23 and 4 C, temperature dependencies almost identical to those of phage f2 RNA (Fig. 6) were observed (figures not shown). Also when rapidly labeled HeLa cell cytoplasmic RNA

FIG. 6. Chromatography of phage f2 RNA at 23 and 4 C. Fractions of 2 ml were taken. The column was 12 mm by 9 cm. The plot is of OD_{260} per ml. In both cases the total amount recovered is obtained by doubling the sum recoveries in every fraction. For the run at 23 C (solid line), the recovery was 7.2 OD_{260} units of an added 5.8 OD_{260} units. For the run at 4 C (dashed line), the recovery was 3.2 OD_{260} units of an added 5.8 units.

obtained by phenol extraction of the cytoplasm after 30 min of labeling with ^3H -uridine was chromatographed on cellulose CF-11 columns at 23 and 4 C, a shift in the elution profile (figure not shown) was obtained almost identical to that of phage f2 RNA shown in Fig. 6.

Effect of temperature on elution profiles of poly U. If the temperature-dependent shift in the elution profiles of the RNA polymers assayed is a function of their secondary structure, then an RNA polymer lacking significant secondary structure would show no temperature dependence on cellulose CF-11 chromatography. Figure 7 depicts an elution profile of poly U run at 4 C. At this low temperature, about 80% of the RNA eluted in the 15% ethanol step at 4 C in contrast to the natural RNA species described above. Poly U has no secondary structure (but does have structure, see reference 13). Consequently, its relative lack of temperature dependence in cellulose CF-11 chromatography supports the hypothesis that the results of this assay reflect the relative amount of secondary structure in the RNA molecule.

FIG. 7. Chromatography of poly U at 4 C. Fractions of 2 ml were taken. The column was 12 mm by 9 cm. The plot is OD obtained on a continuous-flow monitoring device (ISCO, Lincoln, Neb.). In this run about 20% of the optical density eluted in the 35% ethanol step.

DISCUSSION

Chromatography of various single-stranded RNA polymers on columns of cellulose CF-11 at three different temperatures has shown that most of these RNA polymers display altered elution profiles as a function of temperature. At higher temperatures, a larger proportion of the RNA elutes in the 15% ethanol step, and, at lower temperatures, a larger proportion elutes in the 0% step. Extensive data exist showing that RNA polymers which elute in the 15% ethanol step are single-stranded, whereas RNA polymers which elute in the 0% ethanol step are multi-stranded, with double helical portions (5, 9). Based on this temperature dependence, we conjectured that the greater the amount of secondary structure in the various RNA polymers at the time of addition to the column, the greater the probability that they will be eluted in the 0% ethanol peak.

This conjecture has received support from a comparison of Sindbis virus RNA, f2 RNA, and poly U. These three single-stranded RNA polymers have different relative amounts of secondary structure as seen by their melting profiles. Sindbis virus RNA has extensive secondary structure indicated by a sharp, co-operative melting profile (11). Phage f2 RNA, or RNA from R17 which is a serologically related phage, has an intermediate amount of secondary structure as deduced from its broader profile (6). Poly U has no complementary bases and therefore no biologically significant secondary structure; this is reflected in its very low maximal thermal hyperchromicity (13). These three RNA polymers, with their different amounts of secondary structure, chromatograph with correspondingly different elution profiles at varying temperatures. Sindbis virus RNA, with its greater amount of secondary structure, continues to elute in the 0% ethanol step at higher temperatures. Phage f2 RNA, with its intermediate extent of secondary structure, elutes in the 0% ethanol step only at 4 C, whereas at 23 C the same RNA does not elute in the 0% ethanol step. Poly U, completely lacking in biologically significant secondary structure, shows very little elution in the 0% ethanol step even at the lowest temperature tested (4 C).

Thus, the data on the temperature dependence of the elution profile of Sindbis virus RNA, together with the observation of the relationships between the elution profiles of the three RNA polymers studied and their relative amounts of secondary structure, are consistent with the hypothesis that the extent of secondary structure of the molecule at the time of addition to the

column determines whether that molecule chromatographs in the 15% ethanol step or in the 0% ethanol step.

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