Structure of the Ribonucleoprotein of Influenza Virus

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Received for publication 7 June 1972

The ribonucleoprotein (RNP) internal components of influenza virus were separated into distinct size classes by sedimentation in glycerol gradients and examined by electron microscopy by using positive staining with uranyl acetate. The large RNP have a peak in length distribution at 90 to 110 nm, the medium, at 60 to 90 nm, and the small, at 30 to 50 nm. These lengths can be correlated with the estimated molecular weights of the ribonucleic acids contained in the various RNP size classes. The RNP structure appears to consist of a strand which is folded back on itself and coiled in a regular double-helical arrangement.

The exact structure of the ribonucleoprotein (RNP) internal component of influenza virus has not been established. However, there is general agreement that treatment of virions with ether or detergents releases an RNP (12-14, 17, 20, 22, 23, 25-27, 29) which can be resolved into multiple components with different sedimentation coefficients (12, 17, 22, 27). The RNP species separated on the basis of sedimentation in density gradients contain distinct viral ribonucleic acid (RNA) species (12, 17, 23). The isolated RNP contains 10 to 15% RNA (18, 25, 27) as well as multiple copies of a single polypeptide species of about 60,000 daltons (3, 9, 16, 25). Electron micrographs of the isolated RNP showed strands about 15 nm in diameter which ranged from 50 to 130 nm in length (25). The possibility has been considered that such RNP isolated from virions represent fragments of a larger RNP structure (1, 25, 29).

The arrangement of the RNP within the virion is uncertain, primarily because internal structure can be seen only in rare particles by negative staining. An occasional particle shows 6-nm strands which appear to be arranged in a rigid, tight coil about 60 nm in diameter (1, 2), and it has been suggested that all virions contain an internal component of this type. Fixed, sectioned virions do not appear to contain such structures (7, 8, 28), and their appearance is consistent with multiple pieces of RNP in the virion, a result in agreement with most of the studies on isolated RNP and RNA. Examination of spikeless particles by using negative staining with uranyl acetate has also indicated that the rigid, tightly coiled type of internal structure is not found in most influenza virions (28).

Polyacrylamide gel electrophoresis studies of influenza viral RNA indicate that the viral genome is comprised of five to seven segments (4, 6, 11, 24, 30). Recent estimates of the molecular weights of the RNA species indicate that they range from $\sim 3.5 \times 10^5$ daltons for the smallest to $\sim 10^6$ daltons for the largest RNA (4, 10, 19, 30). Evidence has been obtained that different size classes of influenza RNA contain distinct genetic information (10; Horst et al., *J. Mol. Biol.*, *in press*). We have examined the morphology of the RNP in each of the three size classes which were separated by sedimentation on glycerol gradients and correlated the lengths of the RNP with the sizes of the RNA they contain. The observations also provide information about the arrangement of the nucleoprotein strand which forms the RNP.

MATERIALS AND METHODS

Virus. The WSN strain of influenza A virus was used in all experiments and grown in MDBK cells as described previously (5, 10).

Virus labeling and purification. MDBK cells were infected at low multiplicity (0.01 to 0.1) and labeled from 12 to 24-26 hr after infection. For RNP isolation, the virus was labeled with 50 μ Ci of 5'- 3 H-uridine (20 to 25 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per petri dish (10-cm diameter). For the preparation of unfractionated 32 P-viral RNA, the virus was labeled with 2 mCi of $\text{H}_3^{32}\text{PO}_4$ per 10-cm petri dish in phosphate-free 199 medium (GIBCO, Berkeley, Calif.) supplemented with 2% calf serum. Virus was purified as described previously (12), except for the omission of ribonuclease treatment.

RNP isolation and separation on glycerol gradients. 3 H-purified virus was disrupted in low salt buffer (0.02 M NaCl, 0.02 M tris(hydroxymethyl)amino-methane (Tris)-hydrochloride, pH 7.4, 0.002 M ethyl-

enediaminetetraacetic acid [EDTA]) containing a final concentration of 0.5% Triton X-100 and 0.7% sodium deoxycholate and extracted twice with two volumes of ether (9). The disrupted virus was layered on a glycerol gradient (15 to 30%, v/v, in low salt buffer containing 0.05% sodium deoxycholate and prepared over a 0.6-ml cushion of 65% sucrose- D_2O in low salt buffer) and centrifuged for 320 min at 41,000 rev/min in a Spinco SW41 rotor at 5 C.

RNA extraction and analysis. Selected fractions from the glycerol gradient were deproteinized by incubation for 20 min at 37 C with an equal volume of a solution containing 1 mg of Pronase (preincubated for 2 hr at 37 C) per ml in 5 mM Tris-hydrochloride, pH 7.4, 5 mM EDTA, and 0.4% sodium dodecyl sulfate. After addition of 25 μ g of *Escherichia coli* S-RNA, the solutions were made 0.2 M in NaCl, precipitated with two volumes of ethanol, and analyzed by electrophoresis on a 2.5% diacrylate cross-linked polyacrylamide gel following a described procedure (10).

Electron microscopy. Drops from glycerol gradient fractions were applied to grids with carbon-coated Formvar films, rinsed with distilled water, stained with a saturated solution of uranyl acetate, and examined in a Philips EM300 microscope.

RESULTS

Length of influenza RNP. Figure 1 shows a typical radioactivity profile of 3H -uridine-labeled RNP after sedimentation in a glycerol gradient. Fractions labeled I, II, and III were those used for electron microscopy studies. Although no attempt was made to determine sedimentation coefficients in the present study, there is an obvious correspondence of the size classes I, II, and III shown here to the 70S, 60S, and 50S RNP observed by Duesberg (12) after similar detergent treatment and sedimentation on sucrose gradients. Preparations positively stained with uranyl acetate (Fig. 2, 3, and 4) were well-suited for measurements of lengths of the RNP because they were distributed on the grid with little evidence of clumping or structural distortion, whereas aggregation was sometimes noticeable with negative staining. The distinct RNP size classes I, II and III differed in the lengths of the nucleoprotein strands, whereas the width of the RNP strands was 8 to 10 nm in all size classes. Figure 2 shows positively stained RNP from fraction I, with many strands about 100 nm in length. RNP in fraction II (Fig. 3) contain many strands about 80 nm in length, whereas fraction III (Fig. 4) contains shorter strands of about 30 to 50 nm.

The length distribution of RNP was determined by measuring 250 to 300 strands in each size class (Fig. 5). The RNP size class I shows a peak in length distribution between 90 to 110 nm, with only three strands longer than 120 nm and about

20% of the strands shorter than 80 nm. In fraction II, there is a broad peak from 60 to 90 nm, as well as some shorter and longer strands. RNP size class III shows a sharp peak from 30 to 50 nm, with 56% of the strands falling in this range. About 28% of the remaining RNP strands in fraction III are in the 50 to 70-nm size range.

The RNA species present in each RNP size class were analyzed by polyacrylamide gel electrophoresis to ensure that the preparations of RNP which were used for length measurements represented fractions containing distinct RNA and to determine which RNA species were present in each size class. RNP size class I contained primarily the largest RNA species, with some molecules of the intermediate-size RNA and essentially none of the smallest-size RNA. RNP size class II contained intermediate-sized RNA and some of the largest-size RNA. RNP size class III consisted mainly of the smallest RNA and some of the smaller of the intermediate-size RNA. Thus,

FIG. 1. Glycerol gradient sedimentation of ether-detergent-treated 3H -uridine-labeled WSN strain of influenza A virus. The conditions for disruption and glycerol gradient were as described in Materials and Methods. Samples (50 μ liters) of each fraction were counted in a Tri-Carb liquid scintillation counter after addition of 2 ml of a solution containing 20% NCS (Nuclear Chicago Corp.) and 80% toluene-based scintillation fluid. The first arrow (fraction 2) indicates the position of intact or incompletely disrupted virus on the dense 65% sucrose- D_2O "cushion" at the bottom of the gradient. The fractions selected to represent three classes of RNP (12) for electron microscopy and RNA analysis are indicated by I (fraction 7), II (fraction 10), and III (fractions 13 and 14).

FIG. 2. Positively stained RNP from size class I, with many strands about 100 nm in length. $\times 115,000$.

FIG. 3. Positively stained RNP from size class II, showing 60 to 90 nm RNP, as well as a few longer RNP. $\times 115,000$.

FIG. 4. Positively stained RNP from size class III, with most strands measuring 30 to 50 nm. $\times 115,000$.

the RNP preparations used for electron microscopy showed RNA profiles similar to those described previously for the size classes of influenza RNP separated on the basis of sedimentation rate (12, 17, 23).

To determine whether combinations of RNP from different size classes showed a tendency to aggregate, mixtures including RNP from all size classes were prepared and held at 4 or 37 C for periods as long as 24 hr and then were examined in an electron microscope. This procedure did

FIG. 5. Distribution of lengths of influenza virus RNP in each of the three size classes. These results represent cumulative measurements from two preparations of RNP, one of which is described in Fig. 1; the other had a similar sedimentation profile. The total number of strands measured in each histogram are: RNP I, 297; RNP II, 250; and RNP III, 294.

not change the appearance of the RNP, and no indication of end-to-end or side-to-side aggregations of RNP was detected.

Fine structure of influenza RNP. A small percentage of positively stained RNP appeared to be partially unwound, exhibiting a loop at one end of the strand (Fig. 6, a to e). The thickness of the strand in the loop was ~ 5 nm, whereas the thickness of the remainder of the strand was ~ 10 nm. Loops were found on larger RNP (Fig. 6, a to c) as well as smaller RNP (Fig. 6, d and e). Negatively stained RNP sometimes showed possible terminal loops (Fig. 6f) and often showed prominent major grooves at a periodicity of about 15 nm (Fig. 6, g to i) as well as indications of a periodicity of about 7.5 nm (Fig. 6f). These images suggest that the RNP is a double-stranded helical structure over most of its length, except at the terminal loop.

DISCUSSION

The present results indicate that influenza RNP with different sedimentation properties are structures with different lengths, but similar diameters, and that the lengths of the structures have discrete upper and lower limits of about 120 and 30 nm. The observed peaks in length distribution of the three size classes of RNP can be correlated with the molecular weights of the RNA which predominate in each size class. Thus, the large

RNP in RNP I (90 to 120 nm) contain the largest RNA (0.9 to 1.0×10^6 daltons), the intermediate RNP in RNP II (60 to 90 nm) contain intermediate RNA (0.6 to 0.8×10^6 daltons), and the smallest RNP in RNP III (30 to 50 nm) contain the smallest RNA (0.3 to 0.4×10^6 daltons). Based on the reported RNA content of about 10% (25), we estimate that the large RNP (~ 100 nm) which contain $\sim 10^6$ daltons of RNA would contain $\sim 9 \times 10^6$ daltons of protein, or about 150 subunits of 60,000 molecular weight. The observed periodicity of 15 nm indicates that there are 6 to 7 turns of the double helix in an RNP structure of 100 nm. Therefore, we estimate that the number of protein subunits per turn of the double helix is in the range of 20 to 26, half of which would be associated with each strand. The number of nucleotides in 10^6 daltons of RNA is about 3,000, giving about 20 nucleotides per protein subunit in influenza virus RNP.

If one assumes that each RNP contains one RNA molecule, one might expect five to seven distinct RNP species instead of the three resolved here, because influenza virus RNA can be resolved electrophoretically into five to seven components. However, the three classes of RNP described here might be heterogeneous. In particular, class II was found to be heterogeneous (Fig. 5). Thus, the discrepancy between the numbers of influenza virus RNA and RNP resolved might

FIG. 6. Fine structure of influenza virus RNP. The positively stained strands (a to e) show a loop on one end, and the thickness of the strands in the loop is less than that in the remainder of the strand. The loops occur on longer (a to c) as well as shorter (d and e) RNP. The negatively stained RNP (f to i) show prominent major grooves at a periodicity of ~ 15 nm (g to i) as well as indications of a periodicity of ~ 7.5 nm (f). One of the strands (f) shows a small loop at the top. Sections a to e, $\times 200,000$. Sections f to i, $\times 292,000$.

be due primarily to differences in the resolving power of density gradients, used to prepare RNP and polyacrylamide gels, used to prepare RNA.

The present observations, as well as previous studies (25, 29), are compatible with a model (Fig. 7) in which the RNP strands are folded back on themselves, giving a loop at one end, and coiled in a regular double-helical arrangement except at the terminal loop. Schulze et al. (29) previously noted alternate deep and shallow grooves in negatively stained RNP, as well as terminal loops at one or both ends of the structure, and suggested that the RNP structure was a double-stranded helix. Complexes of the RNP protein with polyvinyl sulfate had a similar appearance, indicating that the protein has the property which leads to the formation of looped ends (25, 29). These observations have been extended by the present finding of loops on one end of RNP positively-stained with uranyl acetate, a procedure which stains nucleic acid preferentially (15), suggesting that the RNA is present as a loop at one end of the structure. Although we observed loops on only one end of the strands by this procedure, the observations of Schulze et al. (29) suggested the possibility of closed circular RNP strands, which are not ruled out by our observations because loops may only be detected if some uncoiling has occurred. How-

ever, the lack of closed, circular RNA in the RNP is indicated by the finding of free 5' and 3' termini in influenza virus RNA (19, 31).

Two observations in the present study support the view that the isolated RNP are not direct products of random fragmentation of a single, long RNP structure. First, the lengths of the RNP have a discrete range of sizes, which would seem unlikely if random breakage were occurring. Second, the organization of the RNP with a loop on one end suggests that there is no free RNA terminus at that end of the structure, whereas if fragmentation of RNA were occurring, one would expect termini at both ends. These observations are compatible with the idea that the length of RNP is determined by association of proteins with distinct RNA molecules whose lengths are determined genetically.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grants AI-10884 and AI-05600 from the National Institute of Allergy and Infectious Diseases and CA-11426 from the National Cancer Institute, contract no. NIH-71-2173 from the National Institutes of Health, and contract no. AT (11-1)-3504 from the U.S. Atomic Energy Commission. J. C. was supported by International Postdoctoral Fellowship FW-01561. Part of this work was supported by a grant from the Fondation Rose et Jean Hoguet.

We thank Roberta Taylor and Ann Erickson for excellent technical assistance.

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FIG. 7. Model of the suggested structure for influenza virus RNP based on the present results as well as previous observations (25, 29). Between each turn of the double helix, the shaded areas indicate the major grooves, which are thought to be areas showing greater penetration by the stain. The number of protein subunits per turn of the helix was estimated as described in the text.

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