Isolation and Partial Characterization of Nucleic Acid of Influenza Virus

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The principal ribonucleic acid (RNA) component isolated from purified equine influenza virus has an approximate sedimentation coefficient ($S_{20,w}$) of 21S in sucrose gradient containing 0.1 M NaCl. Three other components of 18S, 14S, and 8S were also detected. All the RNA components have characteristics of single-stranded RNA. The average base composition of the principal RNA components is cytosine, 22.2; adenine, 22.9; guanine, 22.3; and uridine, 32.6. There was no qualitative difference in the RNA isolated from noninfectious virus particles compared to that from infectious virions.

Influenza virus, a myxovirus, exhibits certain unique characteristics not shared by other viruses of the group, e.g., Newcastle disease virus and mumps. These are pleomorphism of virions which appear as spheres or filaments (5, 8), genetic recombination (14, 23), multiplicity reactivation (13), incomplete virus production (26), and sensitivity of the early events of replication to ultraviolet light, actinomycin D, and mitomycin C (6, 17). There is also a difference between influenza and parainfluenza viruses in the size of the viral genome. A large ribonucleic acid (RNA) molecule (49S or 57S) has been isolated from Newcastle disease virus (11, 15), but there has been disagreement about the size of the intact RNA genome isolated from influenza viruses; RNA of varying sizes ranging from 38S to 7S have been reported (2, 10, 18, 22, 24). This study was undertaken to characterize the RNA molecular species isolated from purified influenza virions. The present report confirms the observations of Duesberg and Robinson (12), who have recently shown that the RNA of influenza virus contains several RNA components of sizes ranging from 9S to 18S.

MATERIALS AND METHODS

Viruses. The American (Miami) strain of equine influenza virus was used. To minimize the presence of noninfectious virus, the virus stock was prepared in the following manner: three successive egg passages were initiated intrachorioallantoically with 1 egg infectious unit (iu) per 10-day-old embryonated

chicken egg, and 100 iu from the third passage was inoculated into each egg to make the final virus stock. Chorioallantoic fluid was harvested after 20 hr of incubation at 37 C and contained 10^9 iu and 640 hemagglutinin units/ml. Kimber farm strain cross K-137 chick embryonated eggs were used throughout this study.

The BAI strain A of avian myeloblastosis virus (AMV) was used in some experiments for comparison of viral RNA's (19).

Hemagglutination and infectivity assays. The hemagglutinin titers of influenza virus suspensions were determined with human type O erythrocytes according to the technique previously described (17).

Infectivity was assayed by making serial, 10-fold dilution steps of a virus suspension and inoculating 0.1 ml of each dilution step into each of four 10-day-old chick embryonated eggs. After 48 hr of incubation, each egg was tested for the presence of hemagglutinin in the chorioallantoic fluid. The infectivity titer was calculated from the highest dilution factor causing hemagglutinin formation and the fraction of eggs which developed hemagglutinin at that dilution.

Preparation of radioactive viruses. To prepare radioactive influenza virions, 0.1 ml of virus suspension containing 100 iu, and 1 mc of $^{32}\text{PO}_4$, or 200 μC of ^3H -uridine (^3H -UR; Schwarz Bio Research Inc.), were injected simultaneously into each embryonated egg. After incubation at 38 C for 30 or 36 hr, the infected eggs were chilled for 8 hr at 4 C; then chorioallantoic fluid was harvested and used immediately for isolation of virus.

To prepare ^3H -UR-labeled, incomplete (low infectivity) influenza virions, monolayers of chick embryo fibroblasts in 120-mm plastic culture dishes were infected at an input multiplicity of 50 iu/cell (21) and incubated in 10 ml of modified Eagle's medium containing 5% dialyzed calf serum plus 100 μC of ^3H -UR. After 16 hr of incubation in the

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presence of ^3H -UR, the supernatant fraction was harvested and immediately used for virus purification. Each supernatant fraction contained 320 hemagglutinin units and 10^6 iu in 10 ml.

Tritium-labeled AMV was prepared from supernatant fluids of leukemic chicken myeloblast cultures as described by Robinson and Baluda (19).

Virus purification. Influenza virus was purified by a procedure similar to that used for AMV (4). Potassium citrate was added dropwise to fresh chorioallantoic fluid to a final concentration of 0.15 M. The virus was concentrated and partially purified by two successive sedimentations to an interface between a 65% sucrose- D_2O cushion and a 15% sucrose solution containing K-citrate (0.15 M). Finally, the virus preparation was layered over a linear gradient of sucrose (15 to 70%) containing 0.1 M NaCl, 0.01 M tris-(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 0.001 M ethylenediaminetetraacetate (EDTA) (NTE) and centrifuged at 50,000 rev/min for 2 hr in a Spinco SW 50 rotor. Fractions were collected from the bottom of the tube and analyzed for radioactivity, hemagglutinin, and infectivity. The fractions containing the virus were pooled and used for extraction of nucleic acid. The entire procedure was carried out at 2 to 4 C.

^3H -labeled influenza virus was also purified by hemadsorption and elution (10). Packed human red cells (type O) were added to chorioallantoic fluid to a final concentration of 2% and kept at 4 C with occasional shaking for 1 hr in the presence of cold uridine (100 $\mu\text{g}/\text{ml}$). Red cells were sedimented at 2,000 rev/min for 10 min and washed three times with ice-cold phosphate-buffered saline containing cold uridine. The virus was eluted with receptor-destroying enzyme in phosphate-buffered saline (1:10) at 37 C for 2 hr and concentrated over a 65% sucrose- D_2O cushion. Viral nucleic acid was then isolated.

Extraction of viral RNA. Viral RNA was extracted by a procedure previously described (11). The fractions containing virus were pooled and diluted three times with NTE buffer (pH 8.5). Mercaptoethanol to 1.0%; yeast RNA, 200 μg ; and sodium dodecyl sulfate to 1%, were added successively to the virus preparation. The mixture was kept at room temperature for 5 min; it was then extracted three times (6 min each) by shaking with cold phenol equilibrated with NTE buffer (pH 8.5). The nucleic acid was precipitated from the aqueous phase by adding sodium acetate (pH 5.5) to 1.6% and 2 volumes of absolute ethyl alcohol. It was stored at -20 C.

Velocity sedimentation of RNA in sucrose gradients. After two successive precipitations with ethyl alcohol, the viral RNA was dissolved in 0.2 ml of NTE buffer (pH 7.4) and layered over a sucrose gradient (5 to 20%) containing NTE buffer, pH 7.4 (high-salt gradient). For running in low-salt gradients, the RNA was dissolved in distilled water and the sucrose gradient contained only 0.0005 M Tris-HCl (pH 7.4) and 0.0005 M EDTA. After centrifugation at 49,000 rev/min for 2 hr at 4 C in the SW 50 rotor, fractions were collected from the bottom of each gradient by using a Buchner piercing unit.

RESULTS

Properties of purified virus. Figure 1 shows the distribution of hemagglutinin, infectivity, and radioactivity after the final step of purification of ^{32}P -labeled influenza virus by density equilibrium centrifugation in a 15 to 70% linear sucrose gradient. The peaks of hemagglutinin, infectivity, and radioactivity coincided. A visible band also coincided with these peaks. The sharpness of the band indicates homogeneity of the virus preparation. Infectivity per hemagglutinin at the peak was approximately 10^6 . The density of the sucrose gradient at the peak was 1.21 g/ml. Hemagglutinin at the top of the gradient represents free hemagglutinin or hemagglutinin activity associated with pieces of cell membrane or with disrupted virions.

Properties of viral nucleic acid. The fractions containing hemagglutinin and ^{32}P radioactivity in the sucrose density gradient of Fig. 1 were pooled and nucleic acid was isolated as described in Materials and Methods. The isolated ^{32}P -labeled influenza viral RNA was analyzed by

FIG. 1. Density equilibrium ultracentrifugation of ^{32}P -labeled influenza virus. After two sedimentations to an interface between a 65% sucrose- D_2O cushion and a 20% sucrose solution, the virus preparation was diluted with NTE buffer (pH 7.4) and 1 ml was layered over 4 ml of a preformed sucrose gradient (15 to 70%) containing NTE buffer (pH 7.4). After centrifugation in the Spinco SW 50 rotor at 50,000 rev/min for 2 hr at 2 C, fractions were collected from bottom of tube. A sample of each fraction (25 μl) was diluted in 10 ml of Bray's scintillation fluid (9) and assayed for ^{32}P radioactivity ($\circ$). Another sample (10 μl) was used for assay of hemagglutinin ($\times$), and a third sample (10 μl) was assayed for infectivity ($\bullet$).

velocity sedimentation centrifugation (Fig. 2). Ribosomal RNA (28S) added as a marker was detected by absorbancy at 260 $m\mu$. The major ^{32}P -labeled RNA component contained 95% of the total radioactivity and had a sedimentation coefficient of approximately 18S by comparison of positions with 28S ribosomal RNA. This was checked further by using 18S ribosomal RNA as a marker. The peaks of the radioactivity of ^{32}P viral RNA and of the optical density of 18S cell RNA were very close and often coincided. The radioactivity profile of influenza viral RNA was broader than the optical density of 18S ribosomal RNA, and there was an occasional shoulder in the 14S region. About 5 to 8% of the radioactivity was found sedimenting around 8S (Fig. 2).

In another experiment, influenza virions were purified as in Fig. 1. Nucleic acid was isolated from virus present in the three fractions that precede the peak of radioactivity and compared with the nucleic acid isolated from virus present in the peak and the next two fractions. Both viral RNA preparations were analyzed by velocity sedimenta-

tion in sucrose gradients and were found to be identical. They were quantitatively and qualitatively similar to the viral RNA shown in Fig. 2.

To examine further the effect of the method of purification of virus and of the extraction procedure on the size of the isolated viral RNA, the virus was purified by hemadsorption and elution and RNA was extracted in presence of unlabeled chick embryo fibroblast cells. Again, the peak of viral RNA coincided with the peak of 18S ribosomal RNA. The optical density profile of ribosomal RNA also indicated that the RNA extraction procedure did not degrade the ribosomal RNA.

Finally, to eliminate any possible effect of purification or of extraction procedure on the degradation of viral RNA, ^{32}P -labeled equine influenza virus and ^3H -labeled AMV were purified simultaneously. Influenza virus and AMV banded at densities of 1.21 and 1.17 g/ml, respectively, in the sucrose gradient as determined by infectivity and radioactivity. RNA was extracted, after mixing the fractions containing AMV or influenza virus, and analyzed by sucrose velocity centrifugation (Fig. 3). ^3H -labeled AMV RNA, which has a $S_{20,w}$ of 71S (19), has a sharp peak around fraction 8. The ^{32}P -labeled influenza viral RNA has a sharp peak at fraction 20, corresponding to a sedimentation coefficient of 21S by comparison of its relative position to that of 71S AMV RNA

FIG. 2. Velocity sedimentation centrifugation of ^{32}P -labeled RNA isolated from purified influenza virus. ^{32}P -labeled influenza viral RNA and unlabeled 28S ribosomal RNA, used as sedimentation marker, in 0.2 ml of NTE buffer (pH 7.4) were layered on top of a 5.0-ml linear sucrose gradient (5 to 20%) containing NTE buffer (pH 7.4) and centrifuged for 2 hr at 49,000 rev/min at 4°C in a Spinco SW 50 rotor. Fractions were collected and samples of each were analyzed for absorbancy at 260 $m\mu$ and trichloroacetic acid precipitable radioactivity in presence of carrier yeast RNA (100 μg). The arrow indicates the fraction reached by influenza viral RNA when centrifuged in a sucrose gradient (5 to 20%) containing only 0.0005 M Tris-HCl (pH 7.4) and 0.0005 M ethylenediaminetetraacetate, and analyzed in the same way as above. The solid line represents the absorbancy at 260 $m\mu$, and the broken line, ^{32}P radioactivity.

FIG. 3. Sucrose gradient velocity centrifugation of equine influenza virus (EIV) RNA labeled with ^{32}P and AMV RNA labeled with ^3H -uridine. EIV and AMV were purified simultaneously and their RNA's were extracted together, and centrifuged in sucrose velocity gradients at 36,000 rev/min for 2.25 hr in the Spinco SW 39 rotor. ^{32}P radioactivity (broken line) and ^3H radioactivity (solid line) were determined as in Fig. 2.

in the gradient. There is no ^{32}P -labeled RNA component heavier than 21S. Thus, it appears that by all available criteria, the virus purification and the RNA extraction should not have degraded influenza viral RNA.

To determine more precisely the sedimentation coefficient of influenza viral RNA and its homogeneity, the influenza viral RNA was reprecipitated (Fig. 3, fractions 17–23) and run in a sucrose gradient along with ^3H -labeled 18S ribosomal RNA for 3.5 hr at 50,000 rev/min in a Spinco SW 50 rotor at 4°C. The results are presented in Fig. 4. The peaks of optical density and of radioactivity of 18S ribosomal RNA coincided, and the peak of ^{32}P radioactivity of the influenza viral RNA sedimented before the 18S ribosomal RNA. Influenza RNA has a $S_{20,w}$ of 21S by comparing its relative position with that of 18S ribosomal RNA. The shoulder at 18S was not due to nonspecific binding with 18S ribosomal RNA, as it was also present in the absence of 18S ribosomal RNA. In addition, there was a 14S component which had not been resolved at the lower centrifugation speed (36,000 rev/min for 2.25 hr, Fig. 3).

FIG. 4. Sedimentation profile of equine influenza virus (EIV) RNA and 18S ribosomal RNA in sucrose velocity gradient. ^{32}P -EIV RNA from Fig. 3 was reprecipitated and analyzed along with ^3H -labeled 18S ribosomal RNA in sucrose gradient as in Fig. 3 for 3.5 hr at 49,000 rev/min. ^{32}P (×), ^3H (●), radioactivity and absorbance at 260 $m\mu$ (○) determined from samples of each fraction.

TABLE 1. Base composition of influenza virus RNA^a

RNA prepn	Moles per cent			
	C	A	G	U
Viral RNA.....	22.2 (±1.1)	22.9 (±1.0)	22.3 (±1.2)	32.6 (±0.8)
Ribosomal RNA				
28S ^b	28.0	18.7	33.3	20.0
18S ^b	27.7	19.7	27.7	24.9

^a Base composition of influenza virus RNA was determined by using ^{32}P -labeled 18S viral RNA isolated from purified virus (Fig. 2). ^{32}P -labeled virus was prepared after 36 hr of incubation in embryonated eggs as described in Materials and Methods. Base composition was determined after alkaline hydrolysis and paper electrophoresis. The mean per cent and standard deviation was determined from two separate preparations of viral RNA and four separate electrophoresis runs. Ribosomal RNA was labeled with ^{32}P by exposure of noninfected cells to ^{32}P for 4 hr in absence of actinomycin D, and the 28S and 18S fractions were isolated by velocity sedimentation in sucrose.

^b Single determination

The rate of sedimentation of influenza viral RNA was found to depend on the ionic strength in the sucrose gradient. In 10^{-1} M salt, the viral RNA moved faster than in 10^{-3} M salt. The position of influenza viral RNA centrifuged in a sucrose gradient containing 0.0005 M Tris-HCl (pH 7.4) and 0.0005 M EDTA (10^{-3} M salt) is indicated by an arrow in Fig. 2. This reflects the dependence of the configuration of viral RNA on the charge distribution in its vicinity, a property characteristic of single-stranded RNA. Also, more than 97% of the ^{32}P radioactivity in isolated viral RNA was rendered trichloroacetic acid-soluble by ribonuclease treatment in $2 \times \text{SSC}$ (saline-sodium citrate), a condition in which double-stranded RNA is resistant to ribonuclease treatment (3).

To determine whether the 21S viral RNA is a single, covalently linked polynucleotide or an aggregation of components of smaller molecular weight, ^{32}P -labeled viral RNA was dissolved in buffer (Tris-HCl, pH 7.4, 0.01 M; EDTA, 0.001 M), heated in sealed ampoules at 100°C for 2 min, and quenched in ice water. Before heating, 28S ribosomal RNA was added as marker. The sedimentation coefficient of viral RNA after heating was very similar but slightly lower (17S) than that before heating. This could have been due to an irreversible effect of thermal denaturation. Similar changes in sedimentation coefficient of RNA have been shown to be caused by heating and changes in ionic strength (7). It appears then

FIG. 5. Velocity sedimentation centrifugation of ^3H -labeled RNA isolated from purified incomplete virions. Incomplete influenza virions were produced and purified as described in Materials and Methods. After extraction in presence of yeast soluble RNA carrier, the ^3H -labeled RNA and a 28S ribosomal RNA marker were centrifuged in a sucrose velocity gradient at 50,000 rev/min for 2.5 hr at 4°C in the Spinco SW 50 rotor. The position of the 28S ribosomal RNA marker was determined by absorbancy at 260 m μ . The distribution of ^3H label was determined as in Fig. 2.

that the 21S viral RNA is covalently linked and not an aggregation product of smaller components. The average base analysis of ^{32}P -labeled 21S influenza viral RNA is shown in Table 1. The values are incompatible with Watson-Crick complementary base pairing and indicate single strandedness of this RNA. Influenza viral RNA has a high uridylic acid content (33.6%) and a relatively low guanine plus cytosine content (44.5%). A similar base composition for influenza viral RNA has been reported by Agrawal and Bruening (2) and Duesberg and Robinson (12).

RNA from incomplete virions. Virions having low infectivity were produced in chick embryo fibroblasts infected at high moi as described in Materials and Methods. To examine the nature of the RNA content of such viruses, RNA was

extracted from viral hemagglutinin purified by the same technique as that used for normal virions, and analyzed by velocity sedimentation in a sucrose gradient. The results are presented in Fig. 5. There is no qualitative difference between RNA of noninfectious virus particles compared to that isolated from infectious virions. The relative amounts of 21S, 18S, and 14S are also similar to those exhibited by RNA isolated from infectious virions (Fig. 3).

DISCUSSION

The largest RNA component isolated from purified influenza virus has a $S_{20,w}$ of 21S. This 21S RNA does not appear to be the degraded product of a native viral genome of higher molecular weight. There are two critical steps where the viral RNA might be damaged: (i) purification of virus and (ii) extraction of RNA from purified virus. Virus purified by two different procedures, e.g., density-interface sedimentation followed by density equilibrium centrifugation, or by hemadsorption and elution, yielded RNA's of identical size. Finally, simultaneous purification of equine influenza virus and AMV and extraction of RNA after mixing purified equine influenza virus and AMV yielded high molecular weight AMV-RNA and 21S influenza viral RNA (Fig. 3).

An RNA component with a $S_{20,w}$ of 21S, along with other RNA components, has been observed in the RNA of influenza viruses by other workers (2, 12, 18). A heavy RNA component of about 34S, found by Agrawal and Bruening (2) and Pons (18), seems to be an aggregate of 21S RNA caused by divalent cations as shown by Duesberg and Robinson (12). RNA components smaller than 21S found by other workers (12) have also been observed in this study. These were resolved after extended centrifugation. It is not known whether these components represent native components present in the virion or degraded product of 21S RNA. Davies and Barry (10) have obtained only 18S RNA from influenza virus purified by hemadsorption and elution. However, their RNA preparation had not been centrifuged for a long enough time to resolve other components if any were present.

The viral nature of the 21S RNA isolated from virus is evident. The base composition of uniformly labeled 21S viral RNA is in no way similar to that of 18S ribosomal RNA (Table 1; 2, 20). Evidence to be published elsewhere (D. P. Nayak, *in preparation*) shows that the RNA isolated from purified virus causes displacement of plus (viral) strands from duplexes of plus and minus (complementary) intracellular viral RNA strands produced by annealing, and competes with

single-stranded viral RNA for annealing with complementary strands. Also, ^{32}P -labeled viral RNA anneals with intracellular complementary RNA. Finally, 21S RNA can be dissociated from 18S ribosomal RNA after prolonged centrifugation (Fig. 3).

The 21S viral RNA is single-stranded and exists in coiled form, as is evident from its base composition, ribonuclease sensitivity, and dependence of the sedimentation rate on salt concentration. It does not contain complementary RNA, as was found by its inability to self-anneal. The 21S viral RNA is covalently linked and withstands thermal denaturation to a large extent. However, following heat denaturation, all the hydrogen bonds cannot be restored and the original helical form cannot be fully regained, as shown by its slightly lower sedimentation coefficient (17S) after heating and quenching in low salt. Changes in $S_{20,w}$ caused by changes in the helical configuration of MS2 RNA have been reported by Bishop (7). He found that single-stranded MS2 RNA can exist in three different forms having $S_{20,w}$ of 28S, 20S, or 12S, which are dependent upon heat or salt treatment.

A sedimentation coefficient of 21S for influenza viral RNA was determined with two different markers, AMV-RNA having a $S_{20,w}$ of 71S and 18S ribosomal RNA. Both of these RNA species have well-characterized sedimentation coefficients which have been determined in the analytical ultracentrifuge (19). A 21S single-stranded RNA would have a molecular weight of about 0.9×10^6 according to Spirin's formula (25). It is difficult to account for all the known functions coded for by influenza virus RNA, e.g., replication of RNA, synthesis of hemagglutinin, neuraminidase, and coat antigens in a single RNA molecule of 21S. Therefore, one has to assume that a virion contains more than one RNA molecule, since the average estimation of 0.8% of RNA (1, 16) per virion corresponds to RNA having a molecular weight equivalent of 2×10^6 daltons. A similar conclusion has been reached by other workers (10, 12, 18). Hirst (14) postulated more than one RNA strand per virion to account for the high rate of genetic recombination observed in influenza viruses. The results presented above also indicate that there are several RNA strands in an infectious virion. The manner in which these RNA molecules are associated in the virion is unknown.

There is no qualitative difference between the RNA isolated from incomplete virions and that isolated from infectious virus. However, if there were any quantitative difference in RNA content in the noninfectious particle compared to

that in infectious virions, it could not be detected by this procedure.

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