Estimations of the Molecular Weight of the Influenza Virus Genome

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SUMMARY

The molecular weight of the influenza virus genome was estimated by sucrose density-gradient centrifugation, analytical centrifugation and polyacrylamide gel electrophoresis. The results indicated that the virus contained at least six species of RNA of total molecular weight approximately $3.9 \times 10^6$.

INTRODUCTION

The nucleic acid component of influenza virus consists of several species of single-stranded RNA. Three to seven species have been resolved by sucrose density gradient centrifugation and by polyacrylamide-gel electrophoresis (Duesberg & Robinson, 1967; Pons & Hirst, 1968; Lerner & Hodge, 1969).

Although the molecular weight of the genome is reported to be about 2 to $2.5 \times 10^6$ (Ada & Perry, 1954; Frisch-Niggemeyer & Hoyle, 1956; Pons and Hirst, 1968), recent observations in this laboratory on the potential for polypeptide coding of the virus genome indicated that the molecular weight may be considerably greater. These observations prompted this investigation.

METHODS

Virus. The strain of influenza A virus used, x-31, was described by Kilbourne (1969) and grown in the allantoic cavity of 10-day-old embryonated eggs. Virus labelled by $^{32}P$ was produced by injecting 100 $\mu$C $[^{32}P]$orthophosphate (Radiochemical Centre, Amersham, Buckinghamshire) into each egg 8 hr before infection.

Virus purification. This was as previously described (Skehel & Burke, 1969), except that the virus was initially concentrated from the clarified allantoic fluid by centrifugation for 2 hr at 50,000 $g$ using an MSE 6 x 250 ml. angle rotor, and not by adsorption to, and elution from, fowl erythrocytes. The purity of such virus preparations with regard to protein composition is discussed elsewhere (J. J. Skehel & G. C. Schild, unpublished observations). As judged by the absence of 28 s ribosomal RNA, they contain no cellular RNA contamination either in standard preparations or in preparations labelled with $[^{32}P]$orthophosphate.

RNA extraction and analysis. RNA was extracted from purified virus particles suspended in lithium acetate–acetic acid buffer (pH 5.0, 0.05 M) containing EDTA (0.01 M), by adding lithium dodecyl sulphate to 0.5 %. Lithium dodecyl sulphate was prepared from the sodium salt by ion exchange using Dowex 50 in the lithium form as described by Noll & Stutz (1968). This was performed at either 2°, 23°, 37° or 60°C for 2 to 20 min. Procedures to remove protein from such mixtures are given in the text.

Analyses of the extracted RNA were performed either by sucrose density gradient centrifugation or by polyacrylamide gel electrophoresis. Gradients of 15 to 30 % sucrose were used (Becker & Joklik, 1964), except that lithium chloride and lithium dodecyl sulphate
replaced the sodium salts. Polyacrylamide gels containing from 2 to 5% acrylamide were prepared as by Loening (1967), except that lithium acetate replaced sodium acetate and all gels also contained 1% agarose.

Analyses of RNA were also made in the Spinco model E analytical ultracentrifuge fitted with ultraviolet optics. The camera was replaced by a photoelectric slit scanner and the movement of the sedimenting RNA species was presented on an XY recorder as a plot of percentage transmission against distance (Cox, Gould & Kanagalingam, 1968). The solution was scanned at 4 min. intervals after full speed was reached. The temperature and speed of operation are indicated in the text.

Fig. 1. The distribution of influenza virus RNA in sucrose density gradients. Purified virus grown in the presence of $^{32}P$ was suspended in acetate buffer (0.05 M-lithium acetate; 0.01 M-EDTA, pH 5.0) and mixed with lithium dodecyl sulphate (0.5%) at 37°C for 10 min. The lysate was then layered over a continuous density gradient of sucrose (15% to 30% sucrose in 0.01 M-tris; 0.1 M-lithium chloride; 0.5% lithium dodecyl sulphate 0.0025 M-EDTA, pH 7.4) and centrifuged at 24,000 rev./min for 32 hr at 10°C using the SW 27 rotor of the Beckman L 265 B centrifuge. The RNA marker molecules were *Escherichia coli* ribosomal RNA of 16 s and 23 s, mouse ribosomal RNA of 18 s, reovirus double-stranded RNA of 14, 12 and 10.5 s.

RESULTS

Analysis by sucrose density gradient centrifugation

At low ionic strength a single diffuse peak of RNA of sedimentation coefficient approximately 14 s was observed in sucrose gradients. At higher salt concentrations (0.1 M) three peaks of RNA were observed (Fig. 1). The sedimentation coefficient of the influenza RNA species in each peak was estimated by comparing the rates of sedimentation with those of molecules of known molecular weight: mouse and *Escherichia coli* ribosomal RNAs and the double-stranded genome RNAs of reovirus were used for this purpose. The mean sedimentation coefficient of the molecules in each of the three classes was thus determined as 20, 16 and 13 s.

The relative contribution of the RNAs of each class to the total RNA content of the virus was determined by analysing the radioactive RNA extracted from virus grown in the presence of $^{32}P$. Similar distributions of RNA were obtained as those shown in Fig. 1, and from the number of counts/min. detected in the fractions under each of the three RNA peaks (Table 1) the distribution of radioactivity in the RNA size classes was found to be approximately 10:7:4.
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Analysis of the sedimentation velocity of the RNA species

Following experiments such as that reported in Fig. 1, the RNA from the peak fractions was removed from sucrose solution by ethanol precipitation at \(-20^\circ\). The precipitated RNA was redissolved in 0.1 M-phosphate buffer, pH 6.9, then centrifuged at 56,100 rev./min. in the model E Spinco centrifuge. In these experiments double-stranded RNA of sedimentation coefficient 7.2 s, previously characterized by Cox, Kanagalingam & Sutherland (1970), was added in approximately equal amounts to the material from each sucrose gradient peak to act both as carrier during ethanol precipitation and as an internal marker of known characteristics.

Table 1. Distribution of radioactivity between the three RNA size classes of influenza virus

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Total counts/min. in size class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>930 (10)</td>
</tr>
<tr>
<td>2</td>
<td>4700 (10)</td>
</tr>
<tr>
<td>3</td>
<td>1650 (10)</td>
</tr>
<tr>
<td>4</td>
<td>4300 (10)</td>
</tr>
<tr>
<td>5</td>
<td>1270 (10)</td>
</tr>
<tr>
<td>6</td>
<td>316 (10)</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

Expts. 1 to 4 involved RNA analyses by sucrose density gradient centrifugation. Expts. 5 and 6 refer to analyses by polyacrylamide gel electrophoresis. Virus lysates prepared as described in Fig. 1 were made 5% with respect to sucrose and layered over polyacrylamide/agarose gels. The gels contained 3% acrylamide; 0.15% bisacrylamide; 0.04 M-lithium acetate; 0.02 M-tris, pH 7.4; 0.01 M-EDTA; 0.1% lithium dodecyl sulphate and 1% agarose. Electrophoresis was performed at 4° and 2.5 V/cm. for 14 hr.

The radioactivity contained both in fractions from sucrose gradients and in sections of gels was detected using a Packard scintillation spectrometer Model 3375 by recording Cerenkov irradiation (Clausen, 1968). The numbers in parentheses indicate the ratio of the number of counts/min. in each size class in proportion to that in class 1 taken as 10.

The results indicated sedimentation coefficients for the RNA molecules of class 1, 20 to 21 s; class 2, 16 to 17 s; class 3, 13 to 14 s (Fig. 2), and supported the estimates derived above (Fig. 1).

Analysis by polyacrylamide gel electrophoresis

Advantage was taken in these experiments of the superior resolution of RNA species by polyacrylamide-gel electrophoresis coupled with detection by methylene blue staining. Purified virus particles were lysed at pH 5.0 by the addition of lithium dodecyl sulphate and then applied directly to the gels. The results (Fig. 3) demonstrate that (a) the RNA species fall into three size classes as indicated by density-gradient centrifugation, (b) two species of RNA are resolved in each size class.

Analysis of RNA samples isolated from peak gradient fractions by polyacrylamide gel electrophoresis confirmed that the three size classes observed correspond with those separated by sucrose density gradient centrifugation.

The results of other electrophoretic separations of \(^{32}P\)-labelled RNA (Table 1) supported those obtained from sucrose density gradient analyses, namely that the amount of \(^{32}P\) incorporated into the RNA species of the three size classes was distributed approximately as 10:7:4.
Fig. 2. Sedimentation profiles of the RNA of the three size classes. The major fractions of the three RNA peaks obtained from sucrose density gradients (Fig. 1) were mixed to equal concentration with a solution of double-stranded RNA as sedimentation standard, \( (S_{30,w} = 7.2 \) (Cox et al. 1970)). The RNA was removed from sucrose solution by precipitation in 70 % ethanol for 14 hr at -20° and then redissolved in 0.1 M-phosphate buffer, pH 6.9. The \( s \) value of the double-stranded RNA marker (the more slowly sedimenting material) was determined and the ratio of this value to the accurately estimated \( s \) value, 7.2 \( s \) (Cox et al. 1970) was used to correct the determined \( s \) values for the influenza RNA species. (a) Small RNA species (peak III, Fig. 1): temperature 16.1°, 56,100 rev./min.; correction factor 1.075, corrected \( S_{30,w} = 13.1 \) to 13.5. (b) Medium RNA species (peak II, Fig. 1): temperature 12.7°, 56,100 rev./min.; correction factor 1.059, corrected \( S_{30,w} = 16.7 \) to 16.9. (c) Large RNA species (peak I, Fig. 1): temperature 12.8, 56,100 rev./min.; correction factor 1.059, corrected \( S_{30,w} = 20.7 \) to 21.2.
Fig. 3. Separation of the six species of influenza virus RNA. A polyacrylamide/agarose gel prepared as for Table 1. The gel was stained for 1 hr with 0.2% methylene blue in acetate buffer (0.4 M, pH 4.7) and then de-stained in H₂O (Peacock & Dingman, 1967).
The analyses on polyacrylamide gels also gave estimates of the molecular weights of the RNA species. The estimates shown in Table 2 were obtained by using the method of Bishop, Claybrook & Spiegelman (1967) and taking the ribosomal RNAs from *E. coli* and mouse as molecular weight standards.

Table 2. *Sedimentation and electrophoretic migration of RNA species*

<table>
<thead>
<tr>
<th>Size class</th>
<th>Sucrose density-gradient centrif. (Fig. 1), S~20, w</th>
<th>Analyt. centrif. (Fig. 2), S~20, w</th>
<th>RNA species</th>
<th>Mol. wt × 10^-6 (5 expts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>20.7 to 21.2</td>
<td>21.9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21.4</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>18.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.7</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>16.7 to 16.9</td>
<td>14.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.5</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>13.1 to 13.5</td>
<td>13.5</td>
<td>6</td>
</tr>
</tbody>
</table>

The log. molecular weights of the individual species were determined by linear interpolation against migration distance. The ribosomal RNAs of *Escherichia coli* (11.0 and 5.6 × 10^6) and mouse (16.0 and 7.0 × 10^6) were used as standard molecules and separated on parallel gels. Sedimentation coefficients were obtained from mean molecular weight values (mol. wt.) by the relationship S~20, w = (mol. wt/1100) 0.45.

**DISCUSSION**

The results reported here indicate the following properties for the RNA genome of X-31 influenza virus:

(a) The genome comprises at least six discrete RNA species.
(b) These RNA species fall into three size classes with at least two species in each class.
(c) The molecular weights of these RNA species range from approximately 3.5 × 10^6 to 1.0 × 10^6.
(d) If no more than two RNA species are present in each size class, the total molecular weight of the genome is approximately 3.9 × 10^6.

These observations are in reasonable agreement with previous reports on the number of RNA species in influenza virus, although the pairing of the species is possibly more apparent in the results reported here of the methylene blue-stained acrylamide gels.

However, the estimates of the molecular weight of the genome are considerably larger than those previously reported, possibly because these were mainly derived from considerations of the chemical composition of the virus. For this reason the different methods of molecular-weight determination were pursued, and the agreement between the estimates obtained using these methods is an indication of accuracy.

The total molecular weight of the genome value derived is based on the assumption that each virus particle contains one molecule of each of the six RNA species resolved by polyacrylamide-gel electrophoresis. Although no direct evidence has been obtained to support this, indirect support is given by the reproducibility in eleven determinations of the contribution of the RNA in each size class to the total RNA content, as measured in the experiments with ^32P (Table 1).

Several other procedures were used to remove the protein components, including extraction with phenol or a chloroform-isooamyl alcohol mixture (24:1) in the presence of the ribonuclease inhibitors macaloid or diethylpyrocarbonate. However, although similar RNA components were resolved both on sucrose gradients and on polyacrylamide gels, the yields...
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of RNA from these extractions were only about 20%. Attempts to increase yield efficiency by multiple extractions resulted in the appearance of many RNA species. Since the species observed by these procedures differed in size from experiment to experiment, they were presumably non-specific degradation products.

Recent determinations of the molecular weights of the structural polypeptides of influenza virus indicate a total polypeptide molecular weight of approximately $4.2 \times 10^5$ (J. J. Skehel and G. C. Schild, unpublished observations). Although it is not known that each separated polypeptide species represents a distinct class of primary gene products, the virus particle polypeptides clearly represent a large proportion of the coding potential of the virus genome. Moreover, the virus particle polypeptides are also separated into three classes on the basis of size. This situation is similar to that reported for the RNA and polypeptide components of the reoviruses (Smith, Zweerink & Joklik, 1969), and suggests that the RNA species contained in the influenza virus particle are probably monocistronic messenger RNA molecules.

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REFERENCES


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