Electrophoretic Separation of Influenza Virus Ribonucleoproteins

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SUMMARY

A new procedure for the separation of influenza virus particle ribonucleoproteins (RNPs) by electrophoresis on a slab of polyacrylamide gel resolves five discrete bands. The largest is a triplet containing the three largest RNAs (1 to 3), the intermediate sized RNP is a doublet containing RNAs 5 and 6 and the others each contain a single RNA. In addition, each RNP is composed of NP protein and an amount of M which is independent of the size of the RNP. This suggests that some virus particle M is specifically associated with influenza ribonucleoprotein.

INTRODUCTION

It is often assumed that the eight RNA segments of influenza virus are contained in individual ribonucleoprotein (RNP) complexes (Pons, 1971; Compans et al., 1972; Duesberg, 1969). However, the analysis of RNPs by current methods is subject to a number of limitations. Velocity-gradient centrifugation results in a broad band or, at best, three closely migrating size classes (Duesberg, 1969; Kingsbury & Webster, 1969). Density-gradient centrifugation in caesium chloride (Krug, 1971) or in renografin (Caliguiri & Gerstein, 1978) are only able to separate RNPs on the basis of density, while the former method has the additional disadvantage of requiring a fixation step. An alternative electrophoretic procedure for the analysis of influenza virus RNPs was described by Duesberg (1969) but the resolution was little better than that given by centrifugation procedures.

RNPs are believed to have an important role in the replication of influenza virus. During infection at 4 °C, which allows events to proceed up to viral transcription, influenza virus RNP accumulates in the nucleus, but when cells are warmed to 37 °C the majority of the RNP migrates from the nucleus (Hudson et al., 1978). Newly synthesized RNPs with properties similar to those obtained from disrupted virus particles have been detected in both the nucleus and cytoplasm of infected cells (Duesberg, 1969; Kingsbury & Webster, 1969). Density-gradient centrifugation in caesium chloride (Krug, 1971) or in renografin (Caliguiri & Gerstein, 1978) are only able to separate RNPs on the basis of density, while the former method has the additional disadvantage of requiring a fixation step. An alternative electrophoretic procedure for the analysis of influenza virus RNPs was described by Duesberg (1969) but the resolution was little better than that given by centrifugation procedures.

Further evidence for the involvement of RNPs in replication came from Caliguiri & Gerstein (1978) who identified two classes of RNPs in infected cells by centrifugation through renografin gradients. One of these classes was identical to virion RNP and presumably represents a pool of RNPs destined for incorporation into progeny virus particles. The second class was enriched in the P polyypeptides during the early stages of infection and was thought to consist of replication complexes. The possibility of a control of virus replication mediated through the RNPs has been suggested by Bukrinskaya et al. (1979) from their finding that input virion RNPs are modified in infected cells during the early stages of infection.

The analysis of the nature and possible role of RNPs in virus replication depends on a procedure which can resolve individual RNP structures quickly and handle a large number of
samples simultaneously. We describe here a method of electrophoresis through a slab gel which resolves five RNP size classes. These RNPs are characterized with respect to their RNA and protein content.

**METHODS**

*Virus growth and purification.* Influenza virus A/FPV/Rostock/34 (HavlN1) (FP/R) was used throughout. Procedures for the growth of virus labelled with $^{32}$P or $^{35}$S-methionine have been described previously (Hudson *et al.*, 1978); virus purification was according to the methods of Stephenson & Dimmock (1975).

**Polyacrylamide gel electrophoresis (PAGE) of RNA.** RNA was extracted from purified $^{32}$P-labelled FP/R by mixing with an equal volume of 0.15% (w/v) SDS, 8% (w/v) glycerol, 6 M-urea in electrophoresis buffer (Floyd *et al.*, 1974) and heating at 100 °C for 1 min before loading on to the gel. The electrophoresis procedure was based on that of Floyd *et al.* (1974), with the modifications of Palese & Schulman (1976). Slab gels (40 × 20 cm) contained 2.8% (w/v) acrylamide, 0.13% (w/v) bisacrylamide and 6 M-urea in electrophoresis buffer. The electrophoresis buffer contained SDS (0.2%, w/v) and electrophoresis was at room temperature for 16 h at 200 V. For autoradiography the gel was covered with clingfilm (C. E. Payne & Sons, London, U.K.) and exposed without drying, to Kodirex (Kodak) or Fuji Rx X-ray film (Fuji Photo Film Co, Tokyo, Japan).

**Page of proteins.** Purified $^{35}$S-methionine-labelled FP/R and samples from concanavalin A (Con A) chromatography were treated with 0.25 vol. 10% (w/v) SDS, 25% (w/v) β-mercaptoethanol, 25% (v/v) glycerol in 10 mM-tris pH 7.2, at 100 °C for 2 min before loading on to the gel. Slices cut from RNP gels were minced, soaked with 20 to 30 µl of the above solution for 5 min at room temperature and then heated at 100 °C for 2 min before loading on to the gel in 1% agarose. Proteins were separated by electrophoresis on a 10 to 30% linear polyacrylamide gradient slab gel (Cook *et al.*, 1979) using the buffer system of Laemmli (1970). Gels were washed and dried on to filter paper for autoradiography.

**Page of RNPs.** RNPs were extracted from purified virus contained in low salt buffer (0.2 M-NaCl, 0.02 M-tris pH 7.4, 2 mM-EDTA; Duesberg, 1969), by disruption with 0.2 vol. 5% Nonidet P40 (NP40; BDH), 2.5% sodium deoxycholate (DOC; BDH) followed by incubation at 37 °C for 4 min (Pons, 1971) before loading on to the gel. Gradient slab gels of 3 to 4% or 3 to 5% polyacrylamide were formed from the following solutions: 3% (w/v) acrylamide, 0.14% (w/v) bisacrylamide, 10% (v/v) glycerol, 0.048% (v/v) $N,N,N',N'$-tetramethylenediamine (TEMED), 0.48% (w/v) ammonium persulphate and 4% (or 5%) acrylamide, 0.19% (0.23% for 5% gel) bisacrylamide, 30% glycerol, 0.02% TEMED, 0.2% ammonium persulphate. All solutions were prepared in electrophoresis buffer (0.02 M-sodium acetate, 0.04 M-tris-acetic acid pH 7.2, 1 mM-EDTA; Duesberg, 1969). The gel was poured and allowed to polymerize under water-saturated butanol, according to the procedures of Jeppesen (1974). The butanol was removed and replaced with about 7 ml starter gel (Jeppesen, 1974), containing 3% (w/v) acrylamide, 0.14% (w/v) bisacrylamide, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate, prepared in one-fifth strength electrophoresis buffer. Samples were electrophoresed at a constant current of 30 mA in recirculating electrophoresis buffer containing 0.1% DOC. Those gels which were dried before autoradiography were first washed in running tap water for 20 min to remove glycerol.

**Chromatography through Con A-Sepharose.** Virus glycoproteins were separated from non-glycosylated proteins by chromatography through Con A linked to Sepharose 4B (Pharmacia) using methods based on those of Dimmock *et al.* (1977). Slices taken from RNP gels were minced, mixed with 100 µl 2.5% SDS in phosphate-buffered saline (PBS) and incubated at room temperature for 4 to 5 h. The samples were heated at 100 °C for 1-5 min and dialysed overnight at room temperature against a 50-fold excess of 0.25% SDS, 0.14
Electrophoresis of influenza virus RNPs

...continued electrophoresis resulted in little further migration of the RNP bands. Electrophoresis of SDS-treated FP/R in the RNP gel system showed that free virus RNA migrated close behind the bromophenol blue marker (results not shown). During routine electrophoresis of RNPs, free RNA migrates off the bottom of the gel.

The presence of a gradient of acrylamide concentration in the gels was confirmed by pouring a 3 to 4% gel in the usual way and turning it through 90° before adding the starter gel. Samples of extracted RNPs were loaded and electrophoresed, so that they now migrated across, instead of down, the acrylamide gradient. Band separation was greater at lower

m-NaCl, 0·1 m-tris pH 7·5. The acrylamide gel pieces were removed by centrifugation and the supernatants pooled, heated to 100 °C, cooled and applied to a column of Con A-Sepharose. Proteins eluting from the column were precipitated by the addition of 5 vol. of ethanol plus cytochrome c (0·1 mg/ml) and stored at −20 °C overnight. The precipitated proteins were collected by centrifugation, dried and redissolved in a small volume of 0·1 m-tris pH 7·5, 0·05% SDS for analysis by PAGE.

RESULTS

Electrophoretic separation of RNPs

The RNPs of FP/R could not be resolved on low concentration acrylamide/agarose gels and were only poorly resolved on 2·8% acrylamide gels. The inclusion of 0·1% DOC in the electrophoresis buffer was necessary for the separation of distinct RNP bands, but its presence in the gel before polymerization resulted in loss of resolution. Electrophoresis buffer was recirculated to avoid lowering the pH of the lower buffer tank and the resulting precipitation of DOC which interfered with the migration of the RNPs. Resolution was improved by the use of a gradient of acrylamide concentration 3 to 4% or 3 to 5%, and RNP bands were sharpened by the addition of a starter gel. Using this procedure we have been able to separate five RNP size classes from FP/R labelled with either 32P or 35S-methionine (Fig. 1). In early experiments samples were electrophoresed for approx. 20 h, but later results showed 40 h to be optimal. By this time the RNPs were approaching equilibrium in the gel and continued electrophoresis resulted in little further migration of the RNP bands. Electrophoresis of SDS-treated FP/R in the RNP gel system showed that free virus RNA migrated close behind the bromophenol blue marker (results not shown). During routine electrophoresis of RNPs, free RNA migrates off the bottom of the gel.

The presence of a gradient of acrylamide concentration in the gels was confirmed by pouring a 3 to 4% gel in the usual way and turning it through 90° before adding the starter gel. Samples of extracted RNPs were loaded and electrophoresed, so that they now migrated across, instead of down, the acrylamide gradient. Band separation was greater at lower
Table 1. Relative proportions of RNPs obtained from $^{32}$P-labelled FP/R by electrophoresis on a slab gel*

<table>
<thead>
<tr>
<th>RNP</th>
<th>Ct/min</th>
<th>% of total</th>
<th>% Normalized to RNP e</th>
<th>RNA</th>
<th>Nucleotides in RNA ($\times 10^{-3}$)</th>
<th>Relative molar abundance†</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2650</td>
<td>34.6</td>
<td>2.9</td>
<td>1-3</td>
<td>6.9‡</td>
<td>0.38</td>
</tr>
<tr>
<td>b</td>
<td>1240</td>
<td>16.2</td>
<td>1.35</td>
<td>4</td>
<td>1.8</td>
<td>0.68</td>
</tr>
<tr>
<td>c</td>
<td>1744</td>
<td>22.8</td>
<td>1.9</td>
<td>5, 6</td>
<td>3.0‡</td>
<td>0.57</td>
</tr>
<tr>
<td>d</td>
<td>1102</td>
<td>14.4</td>
<td>1.2</td>
<td>7</td>
<td>1.1</td>
<td>1.00</td>
</tr>
<tr>
<td>e</td>
<td>920</td>
<td>12.0</td>
<td>1.0</td>
<td>8</td>
<td>0.9</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*RNPs were located by autoradiography, excised from the gel, and Cerenkov radiation was measured.
†The molar representation of each RNP band was calculated relative to RNP e from the $^{32}$P content of the RNP bands and from the size of the RNA segments (Sleigh et al., 1979). Values are compared with those for the RNA of FP/R (McGeoch et al., 1976).
‡Values are totals for RNAs 1 to 3 and 5 plus 6 respectively.

Assignment of RNA segments to RNP size classes

The separation of at least five RNP size classes suggested that perhaps the eight RNA segments of influenza virus may each be contained in a single RNP structure. Following electrophoresis and autoradiography the RNP bands derived from a $^{32}$P-labelled preparation of FP/R were excised and Cerenkov radiation was measured. The counts in each RNP have been expressed as a percentage of the total and as a ratio relative to the smallest RNP (Table 1). On this basis, we propose that RNP a may be a triplet and RNP c a doublet; the proposed relationship between RNPs and RNA segments is shown. We have also calculated the relative molar abundance of the RNPs, based on their relative amounts and the length of each RNA segment expressed as the number of nucleotides (Sleigh et al., 1979). The values obtained show the large RNPs to be present in less than equimolar amounts and are in general agreement with the values published for the RNA segments (McGeoch et al., 1976).

An analysis of the individual RNP bands was made following electrophoresis on a preparative RNP gel. A 40 cm 3 to 4% acrylamide gel was poured and overlaid with a 3% starter gel. Samples obtained from a purified preparation of $^{32}$P-labelled FP/R were loaded to give about $10^7$ ct/min/track. Following electrophoresis (30 mA for 40 h), the gel was exposed, without drying, for autoradiography (5 h) and the separated RNP bands cut from the gel were cast directly into the top of a 2.8% acrylamide RNA gel. The RNA pattern (Fig. 2) shows that the large RNPs contained large RNA segments, while the small RNPs contained small RNAs. The distribution of RNA released from the RNPs, as compared with the RNA released from a sample of purified $^{32}$P-labelled FP/R set into a section of 4% acrylamide before loading on to the gel, correlates well with the RNP migration pattern and with the proposed assignments given in Table 1. Slight differences in the migration pattern of RNA segments released from the RNP bands compared with labelled virus probably result from setting virus in 4% acrylamide before electrophoresis, whereas the RNP gel slices were taken from a gradient of acrylamide concentration between 3 and 4%. However, RNPs d and e contained RNAs 7 and 8 respectively, RNP b contained predominantly RNA 4 (as well as some contamination with RNAs 5 and 6 from the closely adjacent RNP c) and RNP c about equal proportions of RNAs 5 and 6. RNAs from RNP a all migrated in the position of RNAs 1 to 3 but were not resolved.
Buffer solutions used in the RNP separation and RNA analysis were saturated before use with diethyl pyrocarbonate. However, the presence of the nuclease inhibitor did not entirely prevent partial degradation of some RNA molecules, leading to the occasional appearance of intermediate bands which did not correspond to the FP/R marker.

**Polypeptide composition of RNPs**

Purified FP/R labelled with $^{35}$S-methionine was treated with NP40 and DOC and approx. $10^6$ ct/min were loaded on to an RNP gel. Following electrophoresis, the gel was exposed for autoradiography without drying and the $^{35}$S-labelled RNPs were cut from the gel by assuming co-migration with $^{32}$P-labelled samples in the flanking tracks. Half of each gel slice was treated as described in Methods and loaded on to a polyacrylamide gel for protein analysis. The RNPs contained two proteins which co-migrated with the NP and M polypeptides of FP/R (Fig. 3). The P proteins were not detected, and may have been removed by the DOC treatment used to extract the RNPs (Inglis et al., 1976). This gel did not adequately resolve NA and NP or HA2 and M and the RNP polypeptides may have been contaminated with glycoproteins. To eliminate this possibility, the proteins in the remainder of each $^{35}$S-labelled RNP gel slice were solubilized, pooled and passed through Con A-Sepharose to selectively remove glycoproteins. Recovery of $^{35}$S counts from the column was 74.5%. The proteins eluted were analysed by PAGE and again two polypeptides were observed, co-migrating with
Fig. 3. PAGE of 35S-methionine-labelled polypeptides from RNPs a to e separated on an RNP gel; an FP/R virus marker is included. Pooled RNPs a to e were also passed through a Con A column before electrophoresis to remove any glycoproteins present (Con A/RNP).

Table 2. Calculation of NP:M ratio of RNPs obtained from 35S-methionine-labelled FP/R

<table>
<thead>
<tr>
<th>RNP</th>
<th>NP (Ct/min)</th>
<th>M (Ct/min)</th>
<th>Ratio NP:M</th>
<th>RNA length (nucleotides × 10⁻³)</th>
<th>NP:M/RNA length</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>3039</td>
<td>460</td>
<td>6.60</td>
<td>2.3*</td>
<td>2.9</td>
</tr>
<tr>
<td>b</td>
<td>2662</td>
<td>633</td>
<td>4.21</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>c</td>
<td>2493</td>
<td>721</td>
<td>3.46</td>
<td>1.5*</td>
<td>2.3</td>
</tr>
<tr>
<td>d</td>
<td>2995</td>
<td>1072</td>
<td>2.80</td>
<td>1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>e</td>
<td>2464</td>
<td>1013</td>
<td>2.43</td>
<td>0.9</td>
<td>2.7</td>
</tr>
<tr>
<td>FP/R†</td>
<td>16465</td>
<td>22728</td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are averages for RNAs 1 to 3 and 5 plus 6 respectively.
† Purified virus particles.

NP and M (Fig. 3). Trace amounts of HA1 were present in the separated RNPs but were removed by Con A chromatography. We therefore conclude that RNPs derived from detergent-solubilized FP/R by electrophoresis contain the NP and M polypeptides and are not appreciably contaminated with virion glycoproteins.

The amount of NP and M present in individual RNP bands was determined by excising the radioactive bands after PAGE on a protein gel. The ratio of NP:M was found to vary (Table 2) and was dependent on the size of the RNP from which the proteins were obtained: the smaller RNPs contained proportionately more M than the large RNPs. Dividing each ratio by the length of the corresponding RNA segment resulted in a constant value, suggesting that there may be a fixed amount of M protein associated with each RNP structure.
DISCUSSION

The separation of influenza virus RNPs by electrophoresis is dependent on their size and not charge, since gels of relatively large pore size (results not shown) failed to resolve the RNPs into separate bands. A similar dependence on particle size for the separation of RNP-type structures has been noted for the electrophoretic analysis of ribosome subunits (Talens et al., 1973). The virus structures separated by the gels were shown to contain both RNA and protein since separate isotopically labelled preparations produced identical migration patterns. Free virus RNA migrates very much faster than the RNPs and is lost from the bottom of the gel.

Analysis of $^{32}$P counts in isolated RNP bands cut from gels suggested that RNPs a and c represent respectively an unresolved triplet and a doublet, and implied that each of the eight genome segments of influenza virus was separately packaged in an RNP structure. Analysis of RNA from the RNP bands is consistent with this conclusion.

FP/R RNPs were found to contain both the major core polypeptide, NP, and smaller amounts of M (matrix) protein. Matrix protein is the most abundant influenza A virus polypeptide (Compans et al., 1970), but in isolated RNPs the proportion of M:NP was reduced by at least threefold from that normally found in intact virions. The ratio of $^{35}$S counts in NP compared to M was found to vary between 2.4 and 6.6 depending on the size of the individual RNP (Table 2). The ratio was proportionately higher for the larger RNPs and our results suggest that there may be a constant amount of M protein associated with a discrete site on each RNP. This would argue against the possibility of non-specific association between M and the RNP structures in this preparation. In some other experiments there was no correlation between the NP:M ratio and RNP size, and it seems likely that the specific binding of M to each RNP may have been masked by additional non-specific association. A close association between matrix and nucleoprotein is inferred from chemical cross-linking studies on intact paramyxovirus virions (Markwell & Fox, 1980) and it is suggested that Sendai virus M protein bound to the nucleocapsid has the function of associating the nucleocapsid with the virus envelope (Shimizu & Ishida, 1975) and to have a role in virus assembly (Yoshida et al., 1976). Our finding of a constant amount of M protein in each FP/R RNP may reflect a direct association between the RNP structures and M protein inside the virus particle and a possible function in virus assembly. Non-specific binding of Sendai virus M to nucleocapsid has been described by Hewitt & Nermut (1977).

The isolation of viral RNPs from infected cells is often complicated by an inability to distinguish between viral and cellular RNPs by velocity-gradient centrifugation. Krug (1971) was able to separate such structures only after fixation and caesium chloride centrifugation of peak fractions from sucrose gradients, while Caliguiri & Holmes (1979) resolved viral and host RNPs using renografin gradients, although the separation obtained was not consistent. Preliminary experiments using the polyacrylamide gel procedure described in this paper suggest that viral RNPs can be isolated directly by electrophoresis of infected cell extracts without any apparent interference from host cell RNPs, thus facilitating the direct analysis and comparison of a number of samples in a single step.

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REFERENCES


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