Binding of Ribonucleic Acids to the RNP-antigen Protein of Influenza Viruses

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SUMMARY

Influenza virus plus and minus strand RNA complexed with RNP-antigen protein quite efficiently, while double-stranded virus RNA was not significantly bound. Sindbis and Newcastle disease virus RNA attached to a much lower degree. A small AMP-rich RNA fraction of uninfected cells also formed a complex with the virus RNP-antigen protein.

INTRODUCTION

The nucleocapsid of influenza viruses contains about 10% RNA. This RNA, which represents the total virus genome, can be replaced by polyanions like polyvinyl sulphate (Pons et al. 1969). If such a non-specific substitution of virus RNA holds true for any polyanion and any RNA in the infected cell, the formation of virus particles with a complete genome could hardly proceed. A selective binding of virus RNA to the protein subunits of the nucleocapsid must therefore take place. This preferential affinity could act as a regulatory principle for plus strand RNA synthesis (Scholtissek & Rott, 1970). We have therefore examined the specificity of the binding of virus RNA to the protein of the nucleocapsid.

METHODS

Virus strains. The different strains of influenza virus and the Newcastle disease virus were the same as those used in a previous study (Scholtissek & Rott, 1969c).

Antigens. The large amount of RNP-antigen required for the experiments was obtained from chorioallantoic membranes infected with fowl plague virus (RNP-CAM). The washed tissue was homogenized (20% v/v) with borate buffer (0·1 M-borate, 0·05 M-NaCl, pH 9·0) and frozen and thawed three times. After removal of coarse debris by low speed centrifugation, the extract was treated with 1/5 vol. trichlorotrifluoroethane (Frigen, Farbwerke Hoechst) in a homogenizer for 5 min. in an ice bath. After centrifugation at 3000 rev./min. for 10 min., the supernatant fluid was shaken briefly with ether to remove the residual fluorocarbon. Fine particles were eliminated from the water phase by centrifugation at 78,000 g for 30 min. This solution, devoid of haemagglutinating activity or of antigenic components of virus haemagglutinin, was applied to a column of DEAE-cellulose pre-equilibrated with borate buffer. The loaded column was washed extensively with the buffer containing 0·1 M-NaCl, and the RNP-antigen was eluted with 0·3 M-NaCl. After dialysis against distilled water, the material was lyophilized. The protein content of this preparation was 75%. From electrophoretic and immunoelectrophoretic patterns its RNP-antigen protein content was roughly estimated to be 10 to 20%. The RNA content of the preparation was less than 1%; it contained no material hybridizing with labelled fowl plague minus strand RNA.
RNP-antigen protein (v) from whole fowl plague virus was prepared by shaking a virus concentrate from allantoic fluid with Tween 80 (1.25 mg./ml.) and 1/2 volume ether for 10 min. at room temperature. Haemagglutinin was removed by adsorption to erythrocytes. This RNP-antigen preparation was treated with RNase (10 μg./ml.) for 18 hr at room temperature. It was dialysed against borate buffer and purified on a DEAE-cellulose column as described above. No RNase activity could be detected in the fractions eluted between 0.1 and 0.3 M-NaCl. The E260/E280 ratio was 0.92. The protein content was 1.12 mg./ml.

Antisera. Four months after surviving an infection with fowl plague virus, a chicken was injected intravenously several times at weekly intervals with 5 to 10 mg. of RNP-antigen. Blood samples were drawn 5 to 7 days after the last injection.

A rabbit was hyperimmunized by intramuscular and subcutaneous injection of RNP-antigen from ether-disrupted virus (Schäfer, 1955) incorporated in Freund’s complete adjuvant. One to several months later, alum-precipitated antigen was injected intraperitoneally, and antiserum was prepared from blood taken 10 days later.

Contaminating antibodies against host material and components of the virus envelope were removed by exhaustive absorption of the sera with normal allantoic fluid and haemagglutinin conjugated to polyaminostyrol, as described previously (Becht, 1968). Haemagglutinin, however, was coupled in borate buffer, pH 8.4, since this component has been found occasionally to be unstable at pH 9.0.

Immunoprecipitation. The optimal amounts of antigen and antiserum to be employed for the precipitation reaction were predetermined for each batch of antigen and serum. Graded amounts of antigen were added to 0.2 ml. of antiserum which was used as such or diluted 1/5. The reaction volume was made up to 1 ml. with phosphate-buffered saline and left at room temperature for 1 hr and at 4° overnight. The resulting precipitates were washed twice and their protein content determined by the Folin procedure (Lowry et al. 1951). The optimal concentrations of antigens and antibodies to be employed in the tests were chosen from the beginning of the equivalence zone.

In most of the experiments, RNA in 2× SSC (0.3 M-NaCl, 0.03 M-Na-citrate) was mixed with the RNP-antigen protein and incubated at 20° for 10 min. Thereafter the calculated amount of anti-RNP-serum was added, the samples were further incubated at 20° for 30 min. and left about 15 hr in the refrigerator. The precipitate was centrifuged down and washed once with 10 ml. of buffered saline. Acid precipitation was done with 6% trichloracetic acid. All precipitates were washed with 6% trichloracetic acid, ethanol and ether, dissolved in 0.2 N-NaOH, transferred to the counting vessels, mixed with Bray scintillator and counted in a Packard scintillation counter (Scholtissek & Rott, 1969a). The radioactivity is expressed as disintegrations/min. Further quantitative details are indicated in the tables or in the text.

Preparation of RNA. Plus strand RNAs of influenza and of Newcastle disease virus were prepared according to Scholtissek & Rott (1969b, c). The fowl plague virus plus strand RNA labelled in vivo with [3H]uridine contained between 5 and 10 μg. virus RNA/ml. For comparison with other samples this RNA was mixed with unlabelled rabbit liver microsomal RNA to give the RNA concentration wanted. Minus strand RNA of influenza viruses was synthesized in an in vitro system using [3H]GTP as radioactive marker. A microsomal fraction was prepared from infected fibroblasts 5 hr after infection and was incubated for 20 min. at 36° in a mixture containing the necessary cofactors and [3H]GTP (Scholtissek & Rott, 1969a). After this the microsomes were centrifuged down and the RNA was isolated by phenol + sodium dodecysulphate from the supernatant fluid (Scholtissek & Rott, 1969c). The RNA was dissolved in 2× SSC to give a final concentration between 0.4 and 0.9 mg./ml. Most of this RNA is unlabelled ribosomal chicken RNA. The absolute amount of virus
minus strand RNA in these preparations is not known. It can roughly be estimated to be less than 1%. Since the sera and even the \( \gamma \)-globulin preparations were not free of RNase, it was not possible to consider the role of the molecular weight in the binding reaction. Between 6 and 8% of the radioactive RNA was double-stranded (Scholtissek & Rott, 1969). The values in the tables were corrected for double-stranded RNA. Labelled minus strand RNA of Newcastle disease virus was synthesized \textit{in vitro} using a cytoplasmic fraction prepared 5 hr after infection (Scholtissek & Rott, 1969b). \([\text{H}]\)RNA of Sindbis virus consisting of virus plus strand RNA was prepared by Dr G. Kaluza in a comparable \textit{in vitro} system. The Sindbis and Newcastle disease virus RNA synthesized \textit{in vitro} was heated above the melting temperature and chilled rapidly. It contained about 1% RNase-resistant RNA.

Labelled cellular RNA was prepared by incubating primary chick fibroblast cells either with 50 \( \mu \)c \([\text{H}]\)uridine/culture for 1 hr or with 0.25 mc \([\text{32P}]\)orthophosphate/culture for 2.5 hr. Total RNA was extracted by the sodium dodecylsulphate-phenol method described by Scholtissek & Rott (1970). Double-stranded RNA was prepared by hybridizing 0.4 \( \mu \)g. RNA containing minus strand RNA of fowl plague virus labelled \textit{in vitro} with 20 \( \mu \)g. of unlabelled fowl plague plus strand RNA in 0.5 ml of 2 x SSC. Any excess of single-stranded RNA was digested with 20 \( \mu \)g. of pancreas RNase for 15 rain. at 20° (Scholtissek, 1969). The amount of double-stranded RNA in this preparation is too low to be determined by measurement of \( \Ep_{260} \) (below 5 \( \mu \)g./ml.). Rabbit RNA was isolated from liver microsomes with phenol + sodium dodecylsulphate.

The RNA preparations used carried the following tritium label:

- Fowl plague plus strand RNA: about \( 1.2 \times 10^{6} \) disintegrations/min./\( \mu \)g.
- Fowl plague minus strand RNA: 346 disintegrations/min./\( \mu \)g.
- Cellular RNA: 1630 disintegretations/min./\( \mu \)g.
- Swine influenza minus strand RNA: 348 disintegrations/min./\( \mu \)g.
- A2 minus strand RNA: 216 disintegrations/min./\( \mu \)g.
- B-Lee minus strand RNA: 30 disintegrations/min./\( \mu \)g.
- Sindbis plus strand RNA: 15 disintegrations/min./\( \mu \)g.
- Newcastle disease RNA: 19 disintegrations/min./\( \mu \)g.

The \textit{hybridization technique} for the quantitative determination of virus plus and minus strand RNA has been described (Scholtissek & Rott, 1970).

**RESULTS**

In a preliminary experiment it was found that small amounts of labelled fowl plague plus strand RNA combine with RNP-antigen protein up to 100%. After being bound to the protein, the RNA was not degraded by RNase present in sera and \( \gamma \)-globulins.

The binding efficiency of different kinds of RNA is listed in Table 1. The nucleocapsid protein, which binds about 10% of its own weight of virus RNA when incorporated into virus particles (Pons et al. 1969), was used in such amounts that saturation had not been reached, and virtually all plus and minus strand RNA was attached. The specificity of this binding was rather limited, however, since Sindbis and Newcastle disease virus RNA were also bound to a certain degree by the fowl plague protein.

Furthermore, even some cellular RNA combined with the RNP-antigen. It could be shown that a particular species of cellular RNA was picked up preferentially by determining the base composition of this RNA fraction. Cellular RNA was labelled with \([\text{32P}]\) for 2.5 hr which corresponds to a 1 hr pulse with \([\text{H}]\)uridine (Scholtissek, 1965). 1-2 mg. of the
labelled RNA was incubated with 2·5 mg. RNP-antigen protein (CAM) in 3·5 ml. 2 × SSC for 10 min., then mixed with 0·8 ml. anti-RNP-serum. Under these conditions, 4% of the labelled RNA was precipitated. The base composition listed in Table 2 indicates that an RNA extremely rich in AMP has been precipitated. Thus a specific fraction of cellular RNA binds to the RNP-antigen protein.

Double-stranded fowl plague RNA does not combine with RNP-antigen protein. The labelled double strands can be recovered without any loss from the supernatant fluid after immune precipitation, as shown in Table 3.

### Table 1. Co-precipitation of labelled RNA from different sources with RNP-antigen protein (v) and antiserum

<table>
<thead>
<tr>
<th>Source of labelled RNA</th>
<th>75 μg. RNA/sample</th>
<th>150 μg. RNA/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fowl plague plus strand</td>
<td>99</td>
<td>—</td>
</tr>
<tr>
<td>Fowl plague minus strand</td>
<td>99</td>
<td>81</td>
</tr>
<tr>
<td>Cellular RNA</td>
<td>20</td>
<td>8·5</td>
</tr>
<tr>
<td>Swine influenza minus strand</td>
<td>—</td>
<td>81</td>
</tr>
<tr>
<td>A 2 minus strand</td>
<td>—</td>
<td>88</td>
</tr>
<tr>
<td>B-Lee minus strand</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>Sindbis plus strand</td>
<td>48</td>
<td>26</td>
</tr>
<tr>
<td>NDV minus strand</td>
<td>36</td>
<td>21</td>
</tr>
</tbody>
</table>

RNP-antigen protein (0·11 mg.) (v) was incubated either with 75 or 150 μg./sample labelled RNA from different sources for 10 min. Thereafter, 0·5 ml. rabbit antiserum was added. In line 1 of the table, about 0·1 μg. labelled fowl plague plus strand RNA was mixed with rabbit RNA to give 75 μg. RNA/sample.

### Table 2. Base composition of 32P-labelled total fibroblast RNA and of the fraction co-precipitated with RNP-antigen protein and antiserum

<table>
<thead>
<tr>
<th>Mole percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
</tr>
<tr>
<td>Total RNA</td>
</tr>
<tr>
<td>Precipitate</td>
</tr>
</tbody>
</table>

### Table 3. Effect of RNP-antigen protein on double-stranded RNA of fowl plague virus

<table>
<thead>
<tr>
<th>Amount of [3H] double strand μl./sample</th>
<th>Disintegrations/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immune precipitate</td>
</tr>
<tr>
<td>25</td>
<td>230</td>
</tr>
<tr>
<td>100</td>
<td>680</td>
</tr>
<tr>
<td>250</td>
<td>1,420</td>
</tr>
</tbody>
</table>

Different amounts of labelled double-stranded RNA were treated with 1 mg. RNP-antigen protein (CAM) in a total volume of 2·5 ml. 2 × SSC for 15 min. at 20°. 0·5 ml. antiserum was used for precipitation. Acid precipitable radioactivity was determined in the immune precipitate as well as in the supernatant. 100 μl. [3H] double-stranded RNA contained 19,800 disintegrations/min.

A further experiment was set up to decide whether both virus strands are bound equally well. As previous investigations had shown, at 3·5 hr after infection about equal amounts of virus plus and minus strands are present in the infected cells (Scholtissek & Rott, 1970). In a set of experiments the appropriate amount of RNP-antigen protein was determined which bound only part of the virus RNA and left some in the supernatant after immuno-precipitation. The following experimental conditions were employed:
Six tissue cultures infected with fowl plague virus received 50 μC [3H]uridine each from 2·5 to 3·5 hr after infection. After phenol extraction, 0·33 mg. of the labelled RNA were mixed with 3·3 mg. RNP-antigen protein (CAM). Precipitation was done with 1·6 ml. chicken antiserum. The RNA was isolated from the immune precipitate as well as from the supernatant fluid. The radioactivity in total RNA and in virus plus and minus strand RNAs was determined according to Scholtissek & Rott (1970). About 60% of the supernatant RNA was degraded during immune precipitation by the RNase activity of the serum. As shown in Table 4, both strands of virus-specific RNA are precipitated. Since the ratio of radioactivity in virus plus and minus strand RNA does not change significantly during immunoprecipitation, it is concluded that plus and minus strand RNA is selected equally well from the mixture.

When a precipitate was produced by reacting an antiserum prepared against normal cellular components with a cell extract, no significant amount (< 1%) of labelled virus RNA was found in the precipitate. Obviously, non-specific trapping of RNA within the immune precipitates did not occur.

Table 4. Precipitation of labelled RNA of fowl plague-infected cells

<table>
<thead>
<tr>
<th>RNA fraction</th>
<th>Disintegrations/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune supernatant</td>
<td></td>
</tr>
<tr>
<td>Total RNA</td>
<td>30,000</td>
</tr>
<tr>
<td>Minus strand</td>
<td>800</td>
</tr>
<tr>
<td>Plus strand</td>
<td>1,250</td>
</tr>
<tr>
<td>Immune precipitate</td>
<td></td>
</tr>
<tr>
<td>Total RNA</td>
<td>27,000</td>
</tr>
<tr>
<td>Minus strand</td>
<td>2,800</td>
</tr>
<tr>
<td>Plus strand</td>
<td>4,500</td>
</tr>
</tbody>
</table>

RNA (0·33 mg.) labelled from 2·5 to 3·5 hr after infection of chick fibroblasts with fowl plague virus in a total volume of 5 ml. 2 × SSC was mixed with 3·3 mg. RNP-antigen protein (CAM) and treated with 1·6 ml. chicken antiserum. RNA was isolated from the immune precipitate as well as from the supernatant and was dissolved in 1 ml. 0·005 M-tris-HCl, pH 8·0, plus 0·001 M-EDTA, each. 0·1 ml. samples were taken for the determination of the radioactivity in total RNA and in virus plus and minus strand RNAs. The latter were determined by hybridization either with maximally 0·5 mg. of RNA containing unlabelled virus minus strand RNA or with maximally 20 μg. of unlabelled virus plus strand RNA, respectively.

DISCUSSION

The results demonstrate that in vitro influenza plus and minus strand RNA complex equally well with the RNP-antigen protein, as long as they are not united as double strands. Although there is a definite selectivity for influenza RNA in the complex formation, it is not exclusively specific, since at least parts of Sindbis and Newcastle disease virus RNA and a fraction of cellular RNA also attaches to fowl plague RNP-antigen protein. No difference in complex formation, however, between minus strand RNAs of influenza A and B viruses has been observed. The in vitro attachment of either strand of virus RNA to the nucleocapsid protein cannot be interpreted as a mere reflexion of synthetic events in the infected cell, because there is no indication of minus strand RNA being enclosed in virus particles. This could be shown by the absence of any self-annealing of labelled RNA isolated from virus particles, even using a concentration of 66 μg./ml. of virus RNA. Under comparable conditions, Newcastle disease and Sendai virus RNA show a high degree of self-annealing which demonstrates that these viruses contain minus and plus strand RNA (Robinson, 1970). If the RNP-antigen protein of influenza viruses has any regulatory function, it might be assumed that this virus protein is synthesized on the plus strand and binds immediately after its synthesis to its template RNA.
That both plus and minus strand RNA complex equally well with the protein is of importance in considering what structural entity of the RNA is recognized by the virus protein. Two complementary RNAs have different base sequences. The tertiary structure caused by intramolecular formation of double-stranded regions is, however, identical in both strands because of their complementary base sequence. Thus, the protein seems to recognize a tertiary structure of the RNA rather than a primary base sequence.

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REFERENCES


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