Newcastle Disease Virus-Specific RNA: Polyacrylamide Gel Analysis of Single-Stranded RNA and Hybrid Duplexes

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Newcastle disease virus-specific [3H]uridine-labeled 18S RNA was resolved by polyacrylamide gel electrophoresis into several components with molecular weights from 450,000 to 840,000. The analysis of 35 and 24S virus-specific RNA also revealed several components in each sedimentational class. The conversion of 18S RNA into double-stranded form by hybridization with an excess of unlabeled virion RNA improved the resolution in polyacrylamide gels and revealed at least six distinct components. The same six classes of hybrid duplexes were revealed when 32P-labeled 50S virion RNA was hybridized with an excess of 18S RNA. The applicability of polyacrylamide gel electrophoresis of hybrid duplexes to the analysis of viral genome structure is discussed.

A large part of virus-specific RNA formed in paramyxovirus-infected cells is represented by molecules of subgenomic size (18 to 35S) complementary to virion RNA (1, 2). Messenger function in the synthesis of virus proteins was tentatively ascribed to the complementary RNA by Kingsbury as early as 1966 (11). Since then a number of data were reported confirming this suggestion. The most abundant class of complementary RNA, 18S RNA, possesses several characteristics which make it a particularly good candidate for the role of paramyxoviral mRNA: a “monocistronic” size (~ 7 × 10^6 daltons), the presence of polyadenylic acid sequences (19), and heterogeneity. The latter was proposed in order to explain the ability of 18S RNA to convert a large part of virion RNA (over 50%, i.e., >3 × 10^6 daltons) into double-stranded form by hybridization (2). Polyacrylamide gel electrophoresis of paramyxovirus-specific RNA revealed a heterogeneity in the region corresponding to 18S RNA, but no individual species were resolved (12, 13).

In this paper we present a series of experiments on polyacrylamide gel analysis of virus-specific RNA isolated from Newcastle disease virus (NDV)-infected cells. Both single-stranded RNA and hybrid duplexes were analyzed. The latter approach was used with a double purpose: first, to improve the resolution of RNA species in the gel; second, as an attempt to find out whether individual species of 18S RNA correspond to specific template regions in the genomic RNA. For the first purpose the hybrids of labeled 18S RNA with an excess of virion RNA were subjected to gel electrophoresis. For the second purpose the products of hybridization of labeled virion 50S RNA with an excess of 18S RNA were analyzed.

MATERIALS AND METHODS

Egg-grown Beaudette strain of NDV (thermostable clone C) and chicken embryo cell (CEC) monolayer cultures were used. The procedures of infection and [3H]uridine-labeling of the cells as well as labeling and purification of the virus, RNA extraction, and rate zonal centrifugation have been described (8).

RNA-RNA hybridization. The procedure described by Kingsbury (11) was used with slight modifications (8). If the product of hybridization had to be analyzed further, the annealing was performed in a 2-ml volume. After annealing, RNase was added (final concentration 10 μg/ml); the sample was incubated at 37°C for 30 min, treated with Pronase (100 μg/ml, 60 min at 37°C) in order to destroy RNase (6), and extracted twice with phenol and precipitated with 2 volumes of ethanol and one-tenth volume of 16% sodium acetate.

Polyacrylamide gel electrophoresis. The procedure described by Schincariol and Howatson (21) was used with some modifications. The gels with acrylamide concentrations of 2.0% or 2.4% (wt/vol) and 0.5% agarose were used. The polymerization was performed at 37°C in tubes 10 cm long with an internal diameter of 0.8 cm. The gels were prerun for 1 h at 6 mA per tube. Ten to 20 μg of RNA in 50 μlitters were layered over the gels. The electrophoresis was performed at room temperature for 3 to 5 h at 6 mA per tube. The gels were either stained with methylene blue or cut with a razor-blade device into slices. The latter were dissolved in 0.2 ml of 30% water at 70°C, mixed with 15 ml of scintillation fluid (2,5-diphenyloxazole, 4 g; 1,4-bis-[5-phenyloxazoly]benzene, 0.1 g; ethanol,

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RESULTS

Preliminary sedimentational fractionation and polyacrylamide gel analysis of single-stranded virus-specific RNA. The distribution of virus-specific RNA in an SDS-sucrose density gradient is shown in Fig. 1. Virus-specific RNA is distributed in three broad peaks: 35, 24 and 18S RNA. Genome-size 50S RNA sediments to the bottom of the tube under these conditions of centrifugation. Such a pattern is characteristic for paramyxovirus-specific RNA extracted from infected cells (1, 2). Peak fractions were pooled as shown in Fig. 1, precipitated with ethanol, extracted with phenol for complete removal of SDS, once more precipitated with ethanol, and applied to polyacrylamide gel. The polyacrylamide gel electrophoresis revealed several components in 35 and in 24S RNA (Fig. 2). Our attention was confined mostly to 18S RNA for reasons discussed above. The analysis of 18S RNA in 2% gel revealed a heterogeneity: two faster moving components formed distinct peaks; the rest were not resolved and formed a slower moving zone (Fig. 3A). The analysis in 2.4% gel revealed six components (Fig. 3C). Component VI in Fig. 3C is represented by a shoulder, but in several experiments it was revealed as a distinct peak. For the calculation of molecular weights of the individual components of virus-specific 18S RNA, we used ribosomal RNAs with known molecular weights as standard markers. We ran 28 and 18S RNA of CEC as well as 23 and 16S RNA of E. coli in parallel tubes and stained them with methylene blue. The molecular weights for 28 and 18S RNA were 1.65 \times 10^6 and 0.67 \times 10^6, respectively (16), whereas for 23 and 16S the values were 1.1 \times 10^6 and 0.53 \times 10^6 (16). The calculated molecular weights of individual NDV-specific 18S RNA components are summarized in Table 1.

Hybridization of the labeled virus-specific 18S RNA with an excess of virion RNA and the analysis of hybrids in polyacrylamide gel. The conversion of single-stranded RNA into double-stranded form by hybridization with complementary strands has been shown to facilitate the resolution of RNA in polyacrylamide gels (7). Annealing of \(^{3}H\)uridine-labeled NDV-specific 18S RNA with an excess of unlabelled virion RNA rendered 95 to 100% of the label ribonuclease resistant. When the product of hybridization after ribonuclease-Pronase treatment (see Materials and Methods) was analyzed in 2% gel, six peaks were consistently resolved (Fig. 4). The components may be tentatively identified with the peaks of single-stranded RNA (Fig. 3C). If the doubled molecular weight of the respective single-stranded

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**PART OF FIGURE 1**

Fractionation of virus-specific NDV RNA by SDS-sucrose density gradient centrifugation. NDV-infected CEC were labeled with \(^{3}H\)uridine from 7 to 10 h postinfection after 2 h of pretreatment with dactinomycin (2 \(\mu\)g/ml). SDS-phenol-extracted RNA was layered on 50-ml 15 to 30% SDS-sucrose density gradient and centrifuged in an SW-25.2 rotor of a Spinco L2 ultracentrifuge at 20,000 rpm for 16 h at 25 C. Fractions were collected and radioactivity was determined in samples (one-twentieth of the fraction volume). Chosen fractions were pooled as shown in the figure (a, b, c) for further analysis. Arrows indicate the position of CEC 28 and 18S ribosomal RNA (28.8 and 17.5 Svedberg units as determined by analytical centrifugation in a Spinco E ultracentrifuge).
component is ascribed to each class of the hybrids, the dependence of their relative mobility on the log of molecular weight seems to be fairly linear (Fig. 5). This confirms to a certain extent the identification of hybrid classes with individual single-stranded components. This argument, however, is based on an assumption that the dependence of relative mobility on log molecular weight described for single-stranded RNA (14) holds also for hybrid duplexes.

Hybridization of labeled 50S virion RNA with an excess of virus-specific 18S RNA and the analysis of hybrids in polyacrylamide gel. Virion RNA of egg-grown Beaudette strain of NDV is represented by molecules with a single polarity and does not produce any significant amount of double-stranded structures when self-annealed (9, 18), differing in this respect from Sendai virus (18, 20). For this reason labeled virion NDV RNA may be used for annealing with an excess of complementary virus-specific RNA for a subsequent analysis of hybridization product.

Labeled virion 50S RNA was obtained either from [32P]-labeled NDV purified by centrifugation through potassium tartrate (8, 10) or from the virus partially purified by differential centrifugation. In the latter case the fractionation of RNA in SDS-sucrose gradient provides a sufficiently pure preparation of 50S RNA (Fig. 6), whereas the loss of virus during purification is negligible.

The hybridization with an excess of unlabeled 18S RNA should convert 50 to 60% of virion RNA into double-stranded form (2). To be sure that 18S RNA was really present in excess, a series of dilutions of 18S RNA preparation was used in every experiment. The amount of 18S RNA in the sample used for further analysis, as shown in Table 2, may be considered saturating. The product of hybridization after ribonuclease treatment had a sedimentation coefficient close to the one expected for the duplex of 18S RNA molecule (Fig. 7). The analysis of the product of annealing in polyacrylamide gel (Fig. 8) revealed the same components as the analysis of hybrids of labeled 18S RNA (Fig. 4).

It should be kept in mind that RNA duplexes in Fig. 8 are represented by fragments of labeled viral genome RNA hybridized with corresponding unlabeled virus-specific RNA. Such fragments may be expected to be present in equimo-
Fig. 3. Electrophoresis of NDV-specific 18S RNA in polyacrylamide gel. A, Total RNA extracted from uninfected CEC labeled for 3 h with [3H]uridine was subjected to electrophoresis in 2% gel for 3.25 h. B, Virus-specific 18S RNA was isolated from sucrose density gradient (Fig. 1, region c) and a part of it was subjected to electrophoresis in 2% gel for 3.25 h. C, 18S RNA was analyzed in 2.4% gel for 4 h.

lar amounts. In an attempt to calculate the molar relations, we ascribed the doubled molecular weight of the corresponding single-stranded component to each class of the duplexes and divided the sum of radioactivity in each peak by the respective molecular weight. The molar relations obtained in three experiments are shown in Table 3. The components III, IV, and V were consistently present in approximately equimolar amounts, whereas the molar relations for components I, II, and VI were lower and more variable.
TABLE 1. Molecular weights of the components of NDV-specific 18S RNA a

<table>
<thead>
<tr>
<th>Component no.</th>
<th>Mol wt* (× 10^6)</th>
<th>No. of determinations</th>
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<tbody>
<tr>
<td>I</td>
<td>446</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>521 ± 9.9</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>608 ± 7.8</td>
<td>6</td>
</tr>
<tr>
<td>IV</td>
<td>696 ± 4.9</td>
<td>6</td>
</tr>
<tr>
<td>V</td>
<td>760 ± 7.2</td>
<td>6</td>
</tr>
<tr>
<td>VI</td>
<td>840 ± 9.7</td>
<td>6</td>
</tr>
</tbody>
</table>

a Three different preparations of virus-specific 3H-labeled 18S RNA were used. Peak I either migrated out of the gel (if an attempt was made to resolve the heavier components) or was poorly resolved. One of the experiments is shown in Fig. 3C.

* Mean ± standard error.

Fig. 4. Polyacrylamide gel electrophoresis of labeled 18S RNA converted into double-stranded form by hybridization with an excess of virion RNA. 3H-labeled virus-specific RNA was isolated as described in the legend to Fig. 1 and annealed with unlabeled virion RNA. A 300-μg portion of total 18S RNA containing 200,000 counts/min of labeled virus-specific RNA was mixed with 290 μg of unlabeled 50S RNA, annealed, and treated as described in Materials and Methods. The annealing rendered ribonuclease-resistant 91% of 3H-18S RNA. The resulting preparation was dissolved in 0.2 ml of electrophoresis buffer. A 0.05-ml portion was applied to 2% gel and run for 4 h.

Fig. 5. A dependence between relative mobility and assumed molecular mass of hybrid duplexes. Relative mobility of hybrid duplexes was calculated from the experiment represented in Fig. 4. Six points correspond to the six peaks resolved in the gel. The individual value of molecular mass for each hybrid component is taken to be equal to the doubled molecular weight of the corresponding single-stranded component (Table 1).

**DISCUSSION**

The heterogeneity of paramyxovirus-specific 18S RNA had been shown with the use of polyacrylamide gel electrophoresis for Sendai virus (12) and for NDV (13), although the number of size classes had not been determined. Rhabdovirus 13S RNA, which is similar to paramyxoviral 18S RNA in many respects, had been shown to consist of several (up to eight) components (21, 22). In the experiments presented in this paper, the resolution power of the gel was increased (7) by conversion of 18S RNA into hybrid duplexes, and the population of 18S RNA was resolved into six distinct components (Fig. 4). The range of the sizes of different classes of RNA, as determined by the analysis of single-stranded 18S RNA preparation (Table 1) roughly corresponds to the expected size of mRNAs for viral proteins (14). The sum of molecular weights of all the components is ~3.4 × 10^6, i.e., ~50 to 60% of the molecular weight of virion RNA (4, 5). This value is in good agreement with the size of the part of viral genome RNA which may be converted into double-stranded form by hybridization with an excess of 18S RNA (2, 8; see also Table 2) and probably serves as a template for 18S RNA synthesis.

It should be taken in mind, however, that the analysis of size distribution of 18S RNA cannot give an answer to the question whether a particular RNA component has an individual base sequence, i.e., whether it is a transcript of...
nucleotide sequences identical to 18S RNA are present in 35S RNA (2).

To study the regions of 50S virion RNA serving as templates for virus-specific 18S RNA, we attempted to cut the labeled 50S RNA into fragments complementary to individual size classes of 18S RNA. The possibility of this approach was not evident a priori: it was based on a presumption of the existence of "spacer" sequences among the template regions in 50S RNA. Such spacers would remain single-stranded and ribonuclease-sensitive after annealing with an excess of 18S RNA. If such spacers were absent, the annealing would produce a population of hybrids containing a 50S RNA molecule as one strand and several 18S RNA molecules stuck end-to-end as the other strand. In this case ribonuclease might be not quite efficient in "cutting" such structures between two adjacent 18S RNA molecules.

The result shown in Fig. 8 indicates that the "cutting" is performed at appropriate sites, because the population of the duplexes (Fig. 8) is similar in the number and size of the components to the one obtained after hybridization of an individual gene, a fragment of a larger transcript, or a transcript of two neighboring individual genes. The latter possibility cannot be disregarded as it had been reported that

![Graph](image-url)
labeled 18S RNA with an excess of virion RNA (Fig. 4).

It should be noted that if each component of 18S RNA is a transcript of an individual template region, the amount of label in hybrid peaks (Fig. 8) should be distributed in equimolar ratios. As one can see from Table 3, this is not generally the case: only the components III, IV, and V are present in equimolar amounts, whereas there is a deficit of the label in the other peaks. The values of molar ratios for the components I, II, and VI were variable, but always less than 1.0, especially for the component VI. The sum of radioactivity in peaks I, II, and VI slightly exceeds the amount needed for the component VI to be present in equimolar relation with components III, IV, and V. One cannot exclude the following possibility: the component VI is a composite of components I and II, i.e., an mRNA-transcript of two neighboring genes. Such a situation could introduce a bias into the equimolar distribution of the label in the population of hybrid duplexes. These considerations indicate that, although the population of 18S RNA is represented by at least six components (Fig. 3, 4), one cannot conclude with certainty whether they are the transcripts of six or five template regions of the genomic RNA.

The data presented in this paper indicate the applicability of polyacrylamide gel electrophoresis of hybrid duplexes for the analysis of the viral genomes which are not fragmented in situ. Further elaboration of the method and its application to DNA-containing viruses might facilitate the study of viral genome structure and function.

![Polyacrylamide gel analysis of hybrid duplexes obtained by annealing of 32P-virion RNA with an excess of 18S RNA. The product of the hybridization experiment described in Fig. 7 was analyzed in polyacrylamide gel. Electrophoresis in 2% gel for 4.25 h.](image)

**Fig. 8.** Polyacrylamide gel analysis of hybrid duplexes obtained by annealing of 32P-virion RNA with an excess of 18S RNA. The product of the hybridization experiment described in Fig. 7 was analyzed in polyacrylamide gel. Electrophoresis in 2% gel for 4.25 h.

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Component no.</th>
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<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>0.52</td>
</tr>
<tr>
<td>3*</td>
<td>0.69</td>
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</table>

*The sum of radioactivity in each peak was divided by the assumed molecular weight of the corresponding component. The value for component VI is taken as 1.0.

*The conditions of annealing and the reaction mixture were similar to those described in the legend to Fig. 7. The fractionation procedure in the gel was as in Fig. 8.

After this paper had been submitted for publication, we became acquainted with a recent work on this subject (3) where virus-specific 18S RNA is resolved into several species in polyacrylamide gel. Our results (Fig. 3C) are in good agreement with the data published by Collins and Bratt.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


