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Separation of the Messenger RNAs of Newcastle Disease Virus by Gel Electrophoresis

(early and late RNA/strain comparisons)

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ABSTRACT **We have separated the 18–22S putative messenger RNA of Newcastle disease virus into seven species ranging in molecular weight from 0.55 to 1.53 × 10^6 using sodium dodecyl sulfate–acrylamide-gel electrophoresis at relatively high concentrations of acrylamide and for a relatively long time. Studies of the number and molecular weights of the proteins and the 18–22S RNAs of the virus suggest that these RNAs are in the right molecular weight range to code for the known proteins of Newcastle disease virus. In preliminary studies using this separation technique, we have demonstrated that: (*a*) there is no difference between the 18–22S RNA made during a normal infection and when genome replication is blocked; and (*b*) there is a strain-specific difference between the RNAs of Newcastle disease virus-AV and Newcastle disease virus-HP.**

Individual monocistronic messenger RNAs (mRNAs) from animal cells have been isolated and characterized from cells that synthesize a limited number of proteins [such as the globin message from erythrocytes (1)] and, with greater difficulty, from cells synthesizing a larger variety of proteins (2). RNA viruses offer another system for analysis of monocistronic mRNAs that function in animal cells. RNA viruses such as reovirus and Newcastle disease virus (NDV) replicate in the presence of actinomycin D, which suppresses host-cell RNA synthesis, thus allowing the detection of virus-specific mRNAs (3–5). In spite of the relatively small number of viral proteins, it is difficult to study the mRNAs as individual species because of their relatively narrow size range. The mRNAs of reovirus have been resolved on Na dodecyl sulfate–acrylamide gels only by annealing the RNAs to the melted strands of the double-stranded segmented genome (6).

Velocity sedimentation in sucrose gradients of the mRNAs of NDV has revealed two species, designated 18 S and 22 S (corresponding to molecular weights of about 0.7 and 1.2 × 10^6), and possibly a third species sedimenting in the range of 35 S (3). Electrophoresis in Na dodecyl sulfate–acrylamide gels has been used to study the RNA synthesized by NDV and by Sendai, another paramyxovirus. These studies (7–9), using gels of low concentration of acrylamide (about 2%) run for short times (1–2 hr), indicated some heterogeneity in the RNA, but did not resolve unique species.

We have separated the mRNAs of NDV into seven species by electrophoresis in Na dodecyl sulfate–acrylamide gels. Our separation technique achieves greater resolution by using a relatively high concentration of acrylamide for this molecular weight range (4%), a long electrophoresis time (11 hr), and a relatively low current (5 mA per gel).

Abbreviations: NDV, Newcastle disease virus; PFU, plaque-forming units.

METHODS AND MATERIALS

Virus-Specific RNA from Infected Cells. Chick-embryo secondary cultures and virus were prepared as described (10). Monolayers (100-mm tissue culture plates with 7 × 10^6 cells) were infected with 5 plaque-forming units (PFU) per cell of NDV-AV (Australia-Victoria-1932). Actinomycin D (5 μg/ml; courtesy of Merck, Sharp & Dohme, Rahway, N.J.) was added 3.25 hr after infection. 45 min later, either 100 μCi/ml of [^{3}H]uridine (28 Ci/mmol, New England Nuclear Corp.) or 15 μCi/ml of [^{14}C]uridine (52.7 Ci/mol, New England Nuclear Corp.) was added. 9 hr after infection, the cell monolayers were solubilized in buffer containing Na dodecyl sulfate, extracted with phenol, and precipitated in alcohol (3). Host-cell RNA was labeled with 2 μCi/ml of [^{14}C]-uridine (52.7 Ci/mol, New England Nuclear Corp.) for about 30 hr.

Cycloheximide Treatment. Virus-specific RNA, synthesized under conditions in which genome replication was blocked, was made by pretreating monolayers with actinomycin D (20 μg/ml) and cycloheximide (50 μg/ml) for 40 min before infection (11). The monolayers were then infected with 50 PFU per cell of NDV-AV and were labeled with 0.2 mCi/ml of [^{3}H]-uridine (28 Ci/mmol, New England Nuclear Corp.) from 0–6 hr after infection.

Sucrose Gradient Sedimentation Analysis of RNA extracts of three 100-mm plates was performed by layering a 0.3-ml sample onto a 12-ml linear 15–30% sucrose gradient in 10 mM Tris·HCl (pH 7.4)–0.10 M NaCl–1 mM EDTA–0.5% Na dodecyl sulfate (11). ^{14}C-Labeled chick-cell RNA was added as marker. The gradient was centrifuged for 4 hr at 180,000 × *g* in a Spinco SW41 rotor at 22°. Samples (0.005 ml) were taken from each 0.4-ml fraction, and trichloroacetic acid-precipitable counts were determined.

Gel Electrophoresis. Labeled virus-specific RNA was subjected to electrophoresis in a 4.0% ethylene-diacrylate cross-linked polyacrylamide gel (10 × 0.6 cm inner diameter) by the procedure of Bishop *et al.* (12) as modified by Duesberg (13). The following makes 25 ml of gel solution: mix well and degas 3.33 ml of a 30% stock solution of recrystallized acrylamide, 8.33 ml of 3 E buffer, 13.06 ml of H_2O, 0.060 ml of ethylene

diacrylate; then add 0.020 ml of TEMED (N,N,N',N'-tetramethylethylenediamine) and 0.2 ml of 10% ammonium persulfate (final concentration 0.8 mg/ml). Electrophoresis was performed at room temperature (25°) with a constant current of 5 mA per gel for 11 hr. After electrophoresis, the gel was frozen and sliced into about 1-mm fractions with a Joyce Loebl gel slicer. The slices were dissolved in 0.1 ml of 1 M piperidine and counted in 5 ml of modified Bray's solution (Kaplan, J. & Moskowitz, M., unpublished). ^{14}C-Labeled chick-cell RNA was added as marker.

RESULTS

Fig. 1 shows the pattern of ^{3}H-labeled virus-specific RNA and ^{14}C-labeled chick-cell RNA after velocity sedimentation in a 15–30% sucrose gradient (11). As described (3, 11, 14), the intracellular virus-specific RNA consists of 50S RNA, which is the same size as virion RNA (15, 16), 35S RNA, which contains some partially base-paired RNA (3, 14), and 18S RNA with a 22S shoulder. This 18–22S RNA is single-stranded [>95% susceptible to digestion by pancreatic and T1 ribonucleases (3, 14)] and is complementary in base sequence to the 50S RNA of the viral genome [becomes resistant to the ribonucleases after annealing with 50S virion RNA (3)]. The 18–22S RNA (and possibly some of the 35S RNA) is considered to be mRNA (17) because most of it is associated with polyribosomes and is released from them by EDTA (3), and because the 18–22S RNA contains poly(A) sequences (Weiss, S. & Bratt, M. A., unpublished).

The 18–22S RNA is resolved by electrophoresis in 4.0% Na dodecyl sulfate–acrylamide gels for 11 hr at 5 mA per gel. Fig. 2 shows the electrophoresis pattern of the viral 18–22S RNAs with ^{14}C-labeled chick-cell RNA added as marker. RNAs *1* and *2* are always distinct peaks. RNA *3* is always the largest peak. RNAs *4* and *5* usually appear as shoulders rather than distinct peaks. RNAs *6–7* appear heterogeneous, but usually with two major peaks. The same gel pattern is obtained with either total virus-specific RNA or 18–22S RNA pooled from a sucrose gradient, since the larger RNAs (50 S and 35 S) do not move into the gel more than the first five slices (5 mm). There is no RNA present at the top of the gel when only the smaller RNAs are electrophoresed (as in Fig. 3).

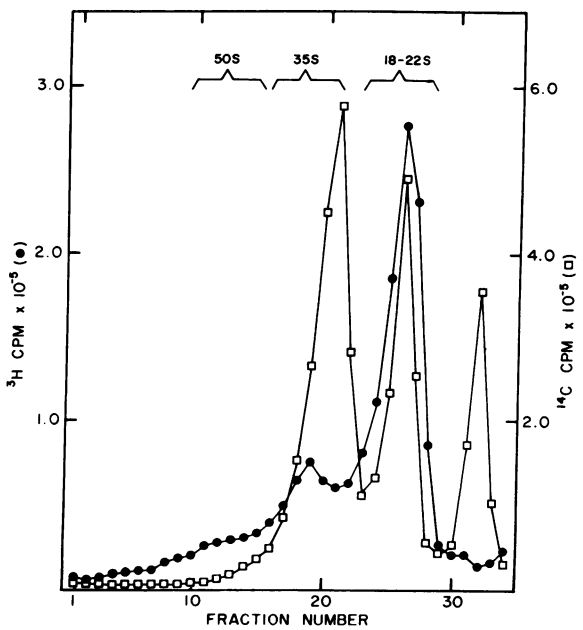

FIG. 1. Sedimentation analysis of NDV-specific RNA. Virus-specific [^{3}H]RNA (●) and chick-cell [^{14}C] RNA (□) were mixed and subjected to sucrose gradient sedimentation analysis. Fraction 1 is the bottom of the gradient.

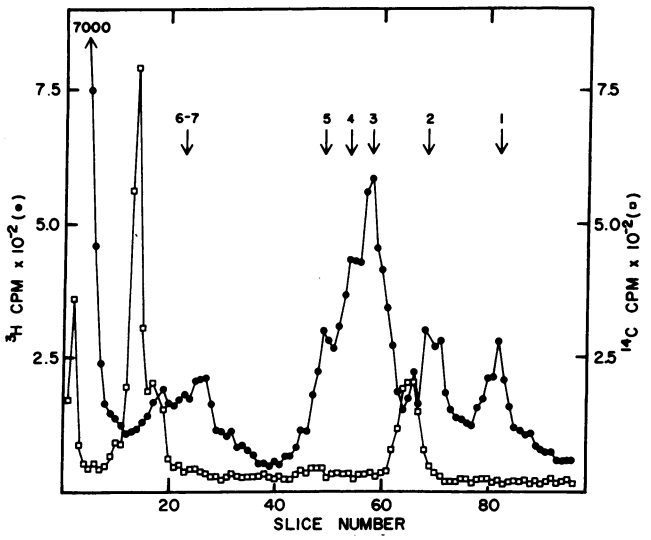

FIG. 2. Na dodecyl sulfate-polyacrylamide-gel electrophoresis of the 18–22S RNAs of NDV. Virus-specific [^{3}H]RNA (●) (from about 1 × 10^{6} infected cells) and chick-cell [^{14}C]RNA (□) were coelectrophoresed. Direction of electrophoresis is from *left* to *right*.

We have also found that, in a sucrose gradient such as that shown in Fig. 1, RNAs *1–7* are distributed through the 18–22S peak on the basis of size. We took fractions 19 through 28 in Fig. 1 and electrophoresed each individually (Fig. 3). Some of the 35S RNA enters the gel but migrates only about 5 mm (the first five fractions) into the gel (not shown). The 22S RNA, fractions 22 and 24 in the sucrose gradient, can be identified as RNAs *6* and *7* in Fig. 3*A* and *B*. RNAs *3*, *4*, and *5* are clearly seen in Fig. 3*B*, *C*, and *D*. RNA *2* appears in increasing amounts in Fig. 3*C*, *D*, and *E*. RNA *1*, the smallest, appears in significant amounts only in Fig. 3*E* and *F*, taken from fractions 27 and 28 on the low-molecular-weight side of the 18S peak in the sucrose gradient. In contrast, the ^{14}C-labeled 18S marker RNA, as expected for a homogeneous species of RNA,* appears as a single peak in slices 57–68 in the gels, between RNAs *2* and *3*, regardless of which fraction in the gradient it was taken from.

In order to determine their molecular weights, the 18–22S RNAs were coelectrophoresed with either the 16S and 23S

* The ^{14}C-labeled 28S ribosomal RNA in Fig. 3*A* (and Fig. 2) does not appear as a single peak, but has a more rapidly migrating component that varies in amount with different preparations of RNA. This more rapidly migrating component appears to be the 28S RNA without the 7S piece of RNA usually associated with it (18), since (*a*) its approximate molecular weight, based on migration in gels, is the same as the molecular weight of the 28S RNA minus the molecular weight of 7S RNA and (*b*) all the 28S RNA is converted to the more rapidly migrating component after heating at 60° for 5 min.

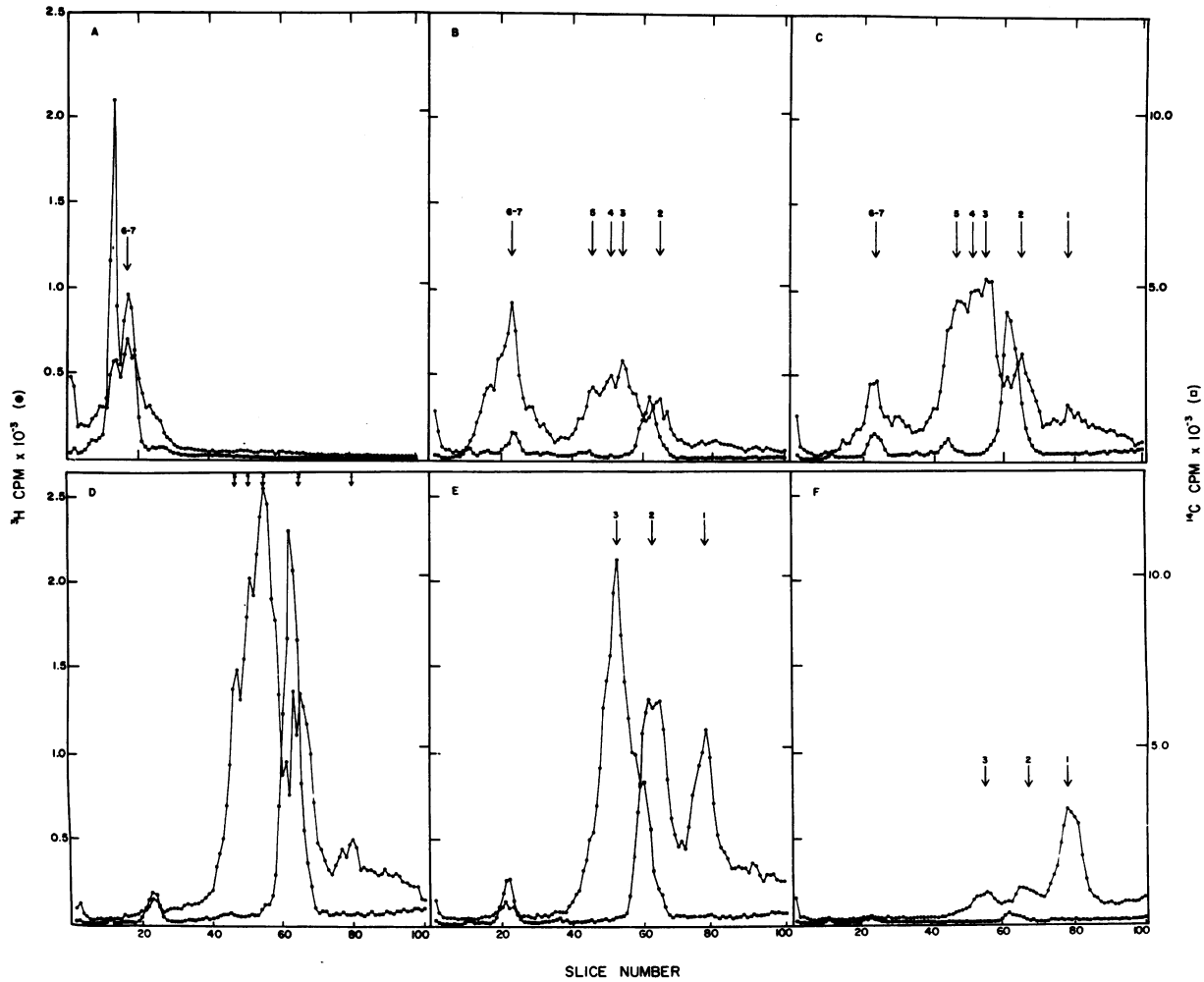

Fig. 3. Na dodecyl sulfate–polyacrylamide-gel electrophoresis of individual fractions of the sucrose gradient in Fig. 1. Samples (0.1 ml) from individual fractions of the sucrose gradient in Fig. 1 were precipitated in alcohol, dissolved in gel sample buffer, and electrophoresed as in Fig. 2. ^{3}H label (●) is virus-specific RNA and ^{14}C label (□) is cell-marker RNA in individual fractions of the sucrose gradient in Fig. 1. (*A*) Sucrose gradient fraction 22; (*B*) fraction 24; (*C*) fraction 25; (*D*) fraction 26; (*E*) fraction 27; (*F*) fraction 28.

ribosomal RNAs of *Escherichia coli* or the 18S and 28S chick ribosomal RNAs. From Amaldi and Attardi's values for the ribosomal marker RNAs (19), the four markers graphed in a straight line when the logarithm of molecular weight was plotted against distance migrated. The approximate molecular weights of the mRNAs, as estimated from this standard curve, are shown in Table 1. Because of the possibility that secondary structure has affected the rate of migration of some of the RNA species, the values in Table 1, ranging from 5.5 to 15.3 $\times$ 10^5, must be considered minimum molecular weight estimates (20). However, no matter what the contribution of secondary structure is to the migration of the 18–22S RNAs, it has allowed us to detect the individual species of RNA.

Applications of the Gel Technique. The gel technique was used to analyze the 18–22S RNA synthesized under different conditions. As a control, the gel in Fig. 4*A* shows the coelectrophoresis of two preparations of RNA from cells infected under similar conditions with NDV-AV, one labeled with ^{3}H and the other with ^{14}C. It indicates that different preparations of RNA labeled with different isotopes are essentially the same. We then determined whether the 18–22S RNA made "late" (during normal infection with NDV) is different from the 18–22S RNA made "early" (when genome replication is blocked). Cycloheximide blocks NDV replication and synthesis of 50S RNA, while allowing synthesis of 35S and 18–22S RNA (11). Fig. 4*B* shows that the 18–22S viral RNA made in the presence of cycloheximide and, therefore, with genome replication blocked (11, 21), contains all the RNA species present late in infection and in the same relative amounts. It is likely that the transcriptase present in the virion is responsible for "early" RNA made during infection in the presence of cycloheximide (11, 22).

The gel technique was also used to compare the RNAs made by NDV-AV and by another strain, NDV-HP (Israel-HP-1935), which makes the same kinds of RNA as NDV-AV—50 S, 35 S, 18–22 S—but less 18–22 S is synthesized relative to 50 S (22). The two strains differ significantly in several biological properties, such as ability to fuse cells (10, 24). In Fig. 4*C*, the RNA patterns of the two strains are similar, but the mobilities of RNAs *1*, *2*, and *3* of NDV-HP are greater than those of NDV-AV; the same results were ob-

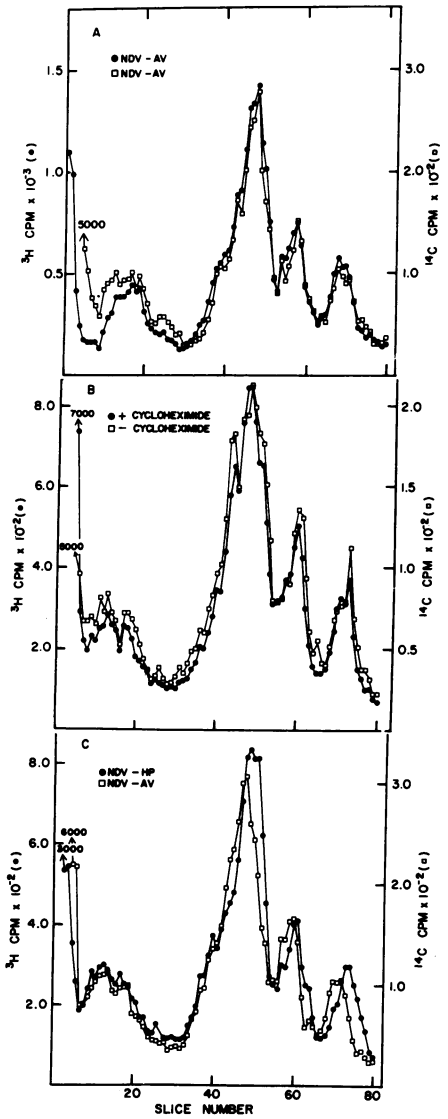

FIG. 4. Na dodecyl sulfate-polyacrylamide-gel electrophoresis of 18-22S RNAs of NDV made under different conditions of infection. (*A*) Coelectrophoresis of two different preparations of RNA from cells infected with NDV-AV, one labeled with ^{14}C (□) and the other with ^{3}H (●). (*B*) Coelectrophoresis of 18-22S RNA (□) from cells infected with NDV-AV as in (*A*) with 18-22S RNA (●) made when genome replication is blocked with cycloheximide. (*C*) Comparison of the 18-22S RNAs from cells infected with NDV-AV (□) coelectrophoresed with 18-22S RNAs from cells infected with NDV-HP (●) and labeled as described in *Methods* for NDV-AV.

tained when the labeling was reversed. The results suggest that these RNAs may be slightly smaller in NDV-HP than in NDV-AV infection.

DISCUSSION

We have separated the 18–22S RNA of NDV into seven species. We are not certain that all the RNA species in the 18–22S region have been separated by our method. However, the gel patterns are qualitatively and quantitatively reproducible for (*a*) different percent gels in the range of 3–5%, (*b*) different durations of electrophoresis from 7–24 hr, (*c*) different amounts of current from 4–10 mA per gel, and (*d*) different preparations of NDV-specific RNA. It seems unlikely that any of the peaks are the result of breakdown or diffusion during electrophoresis because the ^{14}C-labeled 18S and 28S marker RNAs, electrophoresed in the same gels, appear as sharp peaks.

TABLE 1. *Estimated molecular weight of NDV 18–22S RNAs*

RNA species	Estimated molecular weight $\times 10^{-5}$ and standard deviation
16S *E. coli* RNA	5.5
NDV mRNA *1*	5.5 ± 0.3
NDV mRNA *2*	6.8 ± 0.1
18S chick RNA	7.1
NDV mRNA *3*	8.2 ± 0.1
NDV mRNA *4*	9.4 ± 0.2
NDV mRNA *5*	9.9 ± 0.2
23S *E. coli* RNA	11.0
NDV mRNA *6*	13.5 ± 0.7
NDV mRNA *7*	15.3 ± 0.4
28S chick RNA	15.8

NDV-specific RNA was coelectrophoresed in both 3.0% and 4.4% Na dodecyl sulfate–polyacrylamide gels with either *E. coli* 16S and 23S RNAs or chick 18S and 28S RNAs. For each type of gel, a standard curve was constructed by plotting the distance migrated by the four marker RNAs against the logarithm of their molecular weights (19). The molecular weight values of the NDV RNAs were then determined from this standard curve. The values are an average of three determinations.

At this time, our best estimates suggest that NDV-infected cells contain seven or eight virus-specific polypeptides, ranging in size from 33,000–150,000 (Kaplan, J. & Bratt, M. A., in preparation; Hightower, L. & Bratt, M. A., unpublished). Assuming that an mRNA must be at least 10 times the molecular weight of the polypeptide it codes for, the seven mRNAs we have separated are in the correct molecular weight range for the NDV polypeptides.

We have used the gel technique to show that all 18–22S RNA species are made in the same relative amounts both "early" and "late" in infection. In addition, we have detected subtle differences between RNAs made by different strains of NDV.

We have not proven that the RNAs that we have separated are unique species. Competitive annealing studies should determine their uniqueness and their relationship to the genome and the 35S RNA. The best evidence that the RNAs are unique messages would be provided by the *in vitro* translation of an individual RNA species into a specific viral protein.

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