VIROLOGY 77, 772-782 (1977)

Presence of Infectious Polyadenylated RNA in the Coronavirus Avian Bronchitis Virus

GERALD SCHOCHETMAN,[1] ROY H. STEVENS,[2] AND ROBERT W. SIMPSON[3]

Waksman Institute of Microbiology, Rutgers University-The State University of New Jersey, P.O. Box 759, Piscataway, New Jersey 08854

Accepted December 4, 1976

Avian infectious bronchitis virus (IBV) was radiolabeled for its nucleic acid component by growth in deembryonated chicken eggs. Purified virions of IBV have a buoyant density in linear sucrose gradients of 1.17-1.18 g/cm^3 and contain approximately 4% RNA by weight. The genomic ribonucleic acid of IBV shows the following characteristics: (1) It consists of one size class of single-stranded RNA having a molecular weight of 5.5-5.7 $\times$ 10^6 and a sedimentation coefficient of about 48 S; (2) no evidence for subunit structure is apparent since the RNA resolves as a single species with the same electrophoretic mobility in polyacrylamide gels before and after heat denaturation; (3) at least 20-30% of the RNA molecules extracted from purified virions contain sequences of polyadenylic acid of approximately 4 S size; (4) RNA obtained from detergent-disrupted virus particles by phenol-chloroform extraction is infectious for cultures of chick embryo fibroblasts, giving rise to progeny virus which is lethal for embryonated eggs. These findings, together with the observation that IBV virions do not exhibit detectable transcriptase activity, support the conclusion that the genome of this coronavirus acts directly as a messenger RNA in eukaryotic cells.

INTRODUCTION

Infectious bronchitis virus (IBV), originally isolated at this university by Beaudette and Hudson (1937) as the causative agent of a highly contagious respiratory disease of chickens, is presently classified in the recently formed group of riboviruses known as coronaviruses (Tyrrell *et al.*, 1968, 1975). Initial admission of new members to the coronavirus group has been based chiefly on similarities of virion structure such as possession of "club-shaped" surface projections and common features of viral morphogenesis detected in infected cells by electron microscopy. Recent reviews indicate that the diverse viral agents comprising this group are poorly characterized for such properties as their structural proteins, nucleic acid moiety, and molecular mode of biosynthesis (McIntosh, 1974; Kapikian, 1975). A major obstacle which has frequently precluded examination of such properties has been the lack of suitable cell culture systems for producing ample quantities of radiolabeled virus or viral components. This problem is typified by recent studies on coronavirus proteins (Hierholzer *et al.*, 1972; Bingham, 1975) in which it was necessary for investigators to employ optical density tracing methods for electrophoretic resolution of unlabeled virus-derived proteins in polyacrylamide gels.

In this study, we describe the development of an *in vitro* system for propagating relatively high-titered stocks of radiolabeled IBV using a modification of the Bernkopf (1949) deembryonated egg technique. We have previously applied this method for preparing radiolabeled influenza virus (Bishop *et al.*, 1972). Radiolabeled RNA from IBV has been character-

[1] Present address: Frederick Cancer Research Center, Frederick, Maryland 27101.

[2] Present address: Columbia University College of Physicians and Surgeons, School of Dentistry and Oral Surgery, 630 West 168th Street, New York, New York 10032.

[3] Address reprint requests to Dr. Simpson.

ISSN 0042-6822

ized for its strandedness, polarity, and content of polyadenylic acid (poly(A)) sequences. We present the first evidence for the infectious nature of the IBV genome indicating that this avian coronavirus contains an RNA endowed with messenger function. These data are compared with the findings of two recent investigations which are in basic disagreement regarding the nature of IBV virion RNA (Tannock, 1973; Watkins *et al.*, 1975).

MATERIALS AND METHODS

Virus stocks. The highly egg-adapted Beaudette strain of IBV used in an earlier study was employed throughout (Simpson and Groupé, 1959). Working stocks of virus were prepared in 10-day-old white Leghorn embryonated chicken eggs infected by allantoic inoculation with 10^2–10^3 egg LD_{50} (50% lethal doses) and incubated at 37° for 36–48 hr. Storage of infected allantoic fluid stocks was at −70° without additives.

Influenza A virus (WSN strain) and vesicular stomatitis virus (VSV, Indiana strain) grown in primary cultures of chick embryo fibroblasts (CEF) with ^{32}P were also used. ^{32}P-labeled complete (VSV-1) and incomplete (VSV-III) particles of VSV were kindly provided as a gift by Dr. D. H. L. Bishop, who employed the technique described earlier (Bishop and Roy, 1971) for their isolation.

Egg infectivity titrations. Groups of six 10-day-old eggs were inoculated in the allantois with 0.2-ml volumes of assay material diluted by 10-fold steps in BSS buffer with 1% gelatin (GBSS) (Simpson and Hirst, 1961). Embryo deaths occurring during a 7-day incubation period at 37° were recorded, and the 50% lethal dose (LD_{50}) was calculated according to standard methods (Reed and Meunch, 1938).

Preparation of radiolabeled virus. Groups of 57 12-day-old embryonated eggs were inoculated allantoically with approximately 10^6 LD_{50} of IBV diluted in GBSS. The needle holes in the egg shells were thoroughly sealed with a film of collodion, and the eggs were incubated for 2 hr at 37°. Thereafter, each of the eggs was deembryonated from its pointed end in such a manner that the chorioallantoic membrane (CAM) remained adherent to the inner surface of the shell (Bernkopf, 1949). After thoroughly rinsing the CAM with BSS buffer, 7 ml of Eagle's MEM containing 20 μCi/ml of [^{3}H]uridine (20 mCi/mmol) was added, and each egg was covered at the open end with a sterile rubber cap. The eggs were continuously rotated in a special apparatus (Drummond Scientific Co., Broomall, Pennsylvania) for 16 hr at 37° in the chamber of a Wedco humidified CO_2 incubator.

IBV-infected fluids collected from deembryonated eggs were pooled and clarified by centrifugation at 5000 *g* for 20 min. Virus particles were precipitated by treatment with polyethylene glycol as described earlier (Bishop *et al.*, 1972). The precipitate was suspended in STE buffer [0.05 *M* Tris-HCl, 0.1 *M* NaCl, 0.001 *M* ethylenediamine tetraacetate (EDTA), pH 7.2] and sedimented through 5-ml cushions of 30% sucrose STE (w/v) using a Spinco SW 27 rotor run at 25,000 rpm for 90 min. The viral pellets were resuspended in STE buffer, layered on 20-ml linear gradients of 25–80% (w/v) sucrose in STE, and centrifuged in an SW 27 rotor for 16 hr at 22,000 rpm (4°). The 1-ml fractions collected from these gradients were measured for acid-precipitable radioactivity in a Packard Tri-Carb scintillation counter. Egg infectivity of these fractions was determined as described above.

Extraction of RNA from purified virus. IBV particles from pooled fractions of sucrose gradients were disrupted by addition of 1% SDS and an equal volume of extraction buffer (1:1 STE-saturated phenol-chloroform). After two additional extractions with phenol-chloroform, the RNA in the aqueous phase was precipitated overnight at −20° by addition of three volumes of cold 95% ethanol. The RNA precipitate was collected by low-speed centrifugation, resuspended in the appropriate buffer, and used for the various tests described without further storage.

Velocity sedimentation of viral RNA. Aliquots (2 ml) of radiolabeled viral RNA were layered on a 36-ml linear gradient of 15–30% (w/v) sucrose in STES buffer (STE

containing 2% SDS) and centrifuged at 17,000 rpm for 16 hr in an SW 27 rotor at 22°. Each 1-ml fraction collected was measured for its acid-precipitable radioactivity. Alternatively, the viral RNA was layered over an 11-ml linear gradient of 10–30% (w/v) sucrose–STES, centrifuged at 40,000 rpm for 3.5 hr in an SW 41 rotor at 22°, and analyzed for radioactivity as described above.

Resolution of viral RNA by polyacrylamide-gel electrophoresis (PAGE). Electrophoresis of RNA in 2.2% polyacrylamide gels was done for 3.5 hr at 10 mA/gel as described earlier (Bishop and Roy, 1971). The gels were sliced into 1-mm sections, placed in counting vials with 10 ml of toluene-based cocktail containing 3% Protosol (New England Nuclear Co., Boston, Massachusetts), and eluted by mechanical shaking overnight. All samples were subsequently measured for radioactivity in a scintillation counter.

Test for sensitivity of viral RNA to nucleases. Samples of ^{3}H-labeled IBV-RNA dissolved in either 0.3 *M* or 0.01 *M* NaCl buffer (containing 0.01 *M* Tris–HCl, 0.0001 *M* EDTA, pH 7.4) were incubated with 10 μg/ml of RNase A (Worthington Biochemical Corp., Freehold, New Jersey) for 30 min at 37°. Radiolabeled RNA dissolved in 0.01 *M* NaCl buffer (0.005 *M* $MgCl_2$) was incubated for 30 min at 37° with 20 μg/ml of DNase. The amount of acid-precipitable radioactivity present in control and nuclease-treated samples was determined.

Analysis of IBV-RNA for polyadenylic acid content. Purified viral RNA was diluted 30-fold in the appropriate buffer and analyzed for poly(A) sequences by selective adsorption on to cellulose nitrate filters (Lee *et al.*, 1971) or by binding affinity for glass fibers impregnated with polyuridylic acid (poly(U)) (Sheldon *et al.*, 1972).

To estimate the size of poly(A) tracts found in IBV-RNA, the RNA from virus grown in deembryonated eggs with medium containing 20 μCi/ml of [^{3}H]adenosine (30 mCi/mmol) was dissolved in STE and treated at 37° for 30 min with a mixture of pancreatic RNase (5 μg/ml) and RNase T_1 (40 μg/ml). The reaction was terminated by the addition of SDS at 0.5%, final concentration. The digested RNA was layered over a 15–30% (w/v) linear sucrose–STES gradient and centrifuged in a Spinco SW 27 rotor for 20 hr at 25,000 rpm (22°). Gradient fractions were collected and assayed for acid-precipitable radioactivity.

Test for infectious viral RNA. Viral RNA was diluted in chilled high salt buffer (HSB) (0.02 *M* Gomori phosphate buffer, pH 7.2, 0.8 *M* NaCl, and 0.03% EDTA). Confluent monolayers of CEF cells prepared 24 hr earlier by seeding 100-mm petri dishes with 10^8 cells (Simpson and Hirst, 1961) were washed twice with BSS buffer and inoculated with 0.5-ml volumes of RNA. Additionally, 60-mm plates containing one-third this number of cells and inoculated with 0.2-ml volumes were used. Adsorption was carried out at room temperature for 10 min, after which reinforced MEM lacking serum was added to each culture. After incubation at 36° for 2 days, cultures were frozen at −90°, and the resulting cell lysates were clarified by low-speed centrifugation. These lysates were tested for egg lethality by inoculation of undiluted material into the allantois of groups of six 10-day-old eggs. Only deaths occurring after the first day during a 7-day incubation at 37° were included in the final tally.

Test for virion-associated RNA polymerase activity. The assay for viral RNA-dependent RNA polymerase was performed using a standard reaction mixture (0.125 ml) containing 64 m*M* Tris–HCl (pH 8.0), 80 m*M* NaCl, 0.16% Triton X-100, 0.16 *M* dithiothreitol, 0.64 m*M* each of ATP, GTP, and CTP, 0.08 m*M* UTP, [^{3}H]UTP (sp act, 9×10^4 cpm/mmol), and 30 μg of protein of purified test virus (Lowry protein units). Divalent cations were also included either as 8 m*M* $MgCl_2$ or as 0.8 m*M* $MnCl_2$. Reactions were carried out at 33° for 60 min and terminated by addition of approximately 0.15 ml of a saturated phosphate solution (equal parts of $Na_4P_2O_7$ and $Na_3P_2O_4$) and cold TCA at a final concentration of 5%. The precipitates formed during incubation in ice for 15 min were collected on nitrocellulose filters, washed with cold 5% TCA, and measured for radio-

activity by liquid scintillation counting after the filters were dried.

RESULTS

Growth and Purification of Avian Infectious Bronchitis Virus

Preliminary experiments conducted by one of us (R. H. S.) revealed that IBV grown in deembryonated chicken eggs by the method used in this study reaches peak titers in extracellular fluids of about 10^8 egg LD_{50}/ml in 12 hr at 37°. To determine the buoyant density of virus particles, IBV radiolabeled in the presence of [^{3}H]uridine was centrifuged to equilibrium in a linear 25–80% sucrose density gradient (Fig. 1). Coincident peaks for radioactivity and egg infectivity occurred at a density of 1.177 g/cm^3. During the isolation and purification procedure, the progeny virus always behaved as a single, homogenous population. Average yields of 300–500 μg of purified IBV based on protein content (Lowry units) were obtained from groups of 50 eggs, and the RNA-to-protein ratio in these preparations as estimated on a weight basis was 1:25 (i.e., 4% RNA content). RNA measurements were obtained by optical density determinations at 260 nm, assuming that 1 OD unit was equivalent to a concentration of 42 μg/ml. The contribution of the phospholipid components to the total mass of the particle is unknown.

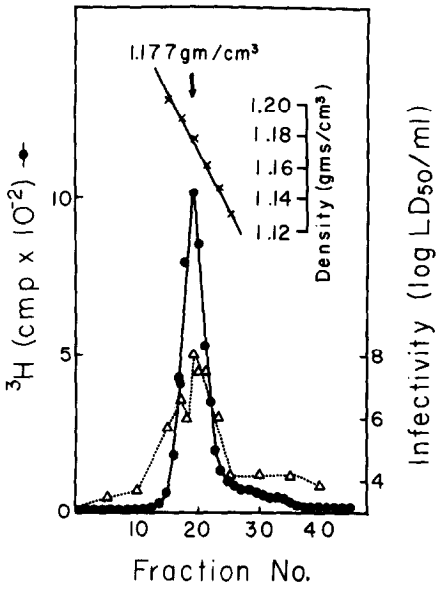

FIG. 1. Buoyant density analysis of avian infectious bronchitis virus. Fifty-seven eggs were inoculated with a 10^{-1} dilution of IBV stock and incubated for 2 hr at 37°. The eggs were deembryonated and were incubated on a rotator at 37° for 16 hr with MEM containing 20 μCi/ml of [^{3}H]uridine. Fluids were harvested and clarified. Virus particles precipitated with polyethylene glycol and pelleted through a sucrose cushion (Materials and Methods) were banded to equilibrium on a 25–80% sucrose gradient in an SW 27 rotor operated at 22,000 rpm for 16 hr. Gradient fractions were tested for acid-insoluble radioactivity and infectivity. Radioactivity (closed circles), infectivity (open triangles).

Characterization of the Virion RNA

Tritiated nucleic acid from purified IBV was analyzed by velocity sedimentation in linear 15–30% sucrose gradients as described in Fig. 2. The ^{3}H-labeled material

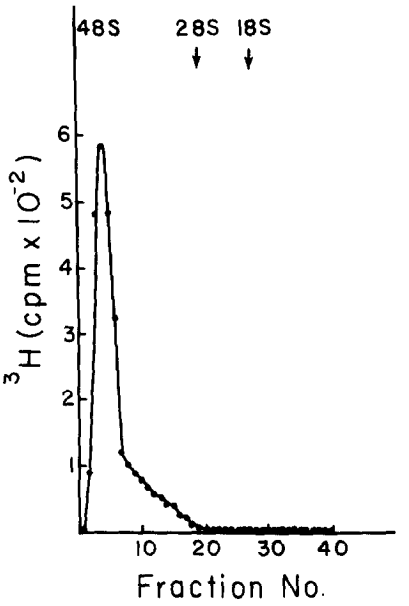

FIG. 2. Velocity sedimentation analysis of RNA extracted from purified IBV. Virus grown in the presence of [^{3}H]uridine was purified by density gradient centrifugation as described in Fig. 1, combined with SDS at a final concentration of 1%, and extracted three times with phenol-chloroform (1:1). Unlabeled HEp-2 cell ribosomal RNA was added as marker, and the RNA mixture was centrifuged through a 15–30% sucrose gradient in STES. Collected gradient fractions were analyzed for optical density values at 260 nm and for acid-precipitable radioactivity.

consistently moved to a position near the bottom of these gradients corresponding to a sedimentation value of approximately 48–50 S relative to the 18 and 28 S ribosomal RNA from HEp-2 cells used as an internal marker. The purified ^{3}H-labeled nucleic acid was also tested for sensitivity to nucleases. Table 1 illustrates the insensitivity of IBV nucleic acid to DNase and its selective degradation by pancreatic RNase at either high or low ionic strength, indicating that the viral genome consists of single-stranded RNA. Using the above data, the molecular weight of IBV-RNA can be calculated as approximately 5.5×10^6 using the formula $MW = 1550\ s^{2.1}$, where MW is the molecular weight and s the sedimentation rate (Spirin, 1962).

Resolution of radiolabeled IBV-RNA in polyacrylamide gels under the conditions described in Fig. 3 revealed a single distinct molecular species. Compared to the relative mobilities of VSV-I and VSV-III RNAs with corresponding molecular weights of 4.4×10^6 and 1.1×10^6, respectively (Bishop and Roy, 1971), the size of IBV-RNA was approximately 5.7×10^6. To determine if the IBV genome RNA was composed of multiple subunits each with a molecular weight less than 5.5×10^6, we measured its electrophoretic mobility after denaturation by heating at 100° for 1 min. The molecule migrated essentially as a single RNA species with approximately the same electrophoretic mobility before and after heat denaturation (Fig. 4).

TABLE 1

SENSITIVITY OF IBV-RNA TO NUCLEASES[a]

Treatment of RNA	Radioactivity (cpm)	Resistance (% control)
None, in 0.3 *M* NaCl	7365	100
RNase in 0.3 *M* NaCl	63	0.9
RNase in 0.01 *M* NaCl	28	0.4
DNase in 0.01 *M* NaCl	7291	90

[a] Tritiated IBV-RNA was dissolved in either high (0.3 *M*) or low (0.01 *M*) salt buffer and digested with RNase (10 μg/ml) for 30 min at 37°. For DNase digestion, IBV-RNA was dissolved in low salt buffer containing 0.005 *M* $MgCl_2$ and digested above with DNase at 20 μg/ml. The amount of acid-precipitable radioactivity in control and nuclease-treated samples was determined by scintillation counting.

Assay for Virion-Associated Polymerase Activity

When IBV was tested for the presence of RNA-dependent RNA polymerase activity under different ionic conditions, essentially no incorporation of radiolabeled uridine 5′-monophosphate could be detected (Table 2). Under appropriate conditions optimal for the *in vitro* transcription of each virus, both VSV and WSN influenza virus particles actively stimulated incorporation of radioactive precursor in these reactions. We can also report that purified virions of IBV do not exhibit an RNA-dependent DNA polymerase activity (data not shown).

Infectivity of IBV Virion RNA

Preliminary experiments done earlier at another institution had suggested that

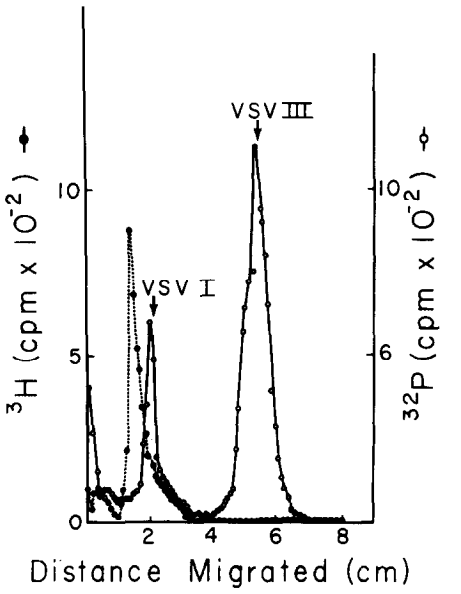

FIG. 3. Polyacrylamide-gel electrophoresis (PAGE) of IBV-RNA. ^{3}H-labeled IBV-RNA, extracted as described in Fig. 2, was mixed with both ^{32}P-labeled VSV-I RNA and VSV-III RNA and subjected to electrophoresis for 3.5 hr at 10 mA/gel through 2.2% polyacrylamide. The gel was sliced into 1-mm sections which were dissolved and counted for radioactivity. [^{3}H]IBV-RNA (closed circles), [^{32}P]VSV-I and [^{32}P]VSV-III (open circles).

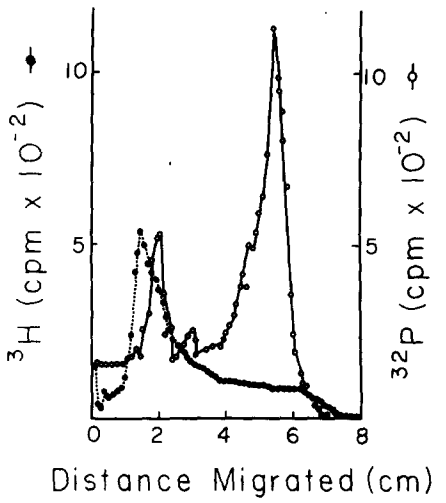

FIG. 4. Heat denaturation of IBV-RNA. An aliquot of the ^{3}H-labeled viral RNA used in the experiment represented in Fig. 3 was mixed with ^{32}P-labeled VSV-I RNA and VSV-III RNA, heated for 1 min at 100°, and analyzed by PAGE. [^{3}H]IBV-RNA (closed circles), [^{32}P]labeled VSV-RNAs (open circles).

TABLE 2

ASSAY FOR RNA-DEPENDENT RNA POLYMERASE ACTIVITY WITH PURIFIED AVIAN BRONCHITIS VIRUS[a]

Test virus	Divalent cations present	[^{3}H]UMP uptake (cpm/60 min)
IBV	Mg^{2+}	50
	Mn^{2+}	45
VSV	Mg^{2+}	90,000
WSN	Mn^{2+}	3,665

[a] Standard reaction mixtures (Materials and Methods) with 30 μg of purified virus (protein units) contained either 8 m*M* $MgCl_2$ or 0.8 m*M* $MnCl_2$ as a source of divalent cations. All tests were incubated at 33° for 1 hr.

phenol-extracted RNA from IBV particles was infectious for cultures of CEF cells but was not infectious when directly inoculated into embryonated eggs (Simpson and Pons, unpublished observations). To pursue this finding further, viral RNA extracted from purified IBV (Materials and Methods) and diluted in hypertonic salt buffer was used to infect primary CEF cultures. In the absence of an efficient plaque assay system, it was necessary for us to test for production of infectious progeny virus in the RNA-treated cultures by measuring for mortality in eggs subsequently inoculated with the cell lysates obtained. The experiment summarized in Table 3 shows that IBV-RNA can generate infectious egg-lethal particles under the conditions employed. The scattered egg mortality pattern observed relative to the original RNA input used to infect CEF cells may possibly reflect nucleic acid interference effects or, alternatively, production of defective interfering particles. The infectivity of our RNA preparations was consistently abolished by treatment with pancreatic RNase but not DNase. CEF cultures originally exposed to RNA inputs of 0.08 μg or higher generally produced 2-day virus yields of about 10^4 to 10^5 egg LD_{50}/ml, which is indicative of the low efficiency of

TABLE 3

TEST FOR IBV-RNA INFECTIVITY IN CEF CULTURES BASED ON PRODUCTION OF EGG-LETHAL PROGENY VIRUS[a]

Inoculum for CEF cells	Response of eggs inoculated with cell lysates	
	D/T[b]	Mortality (%)
IBV-RNA		
$10^{-0.5}$[c]	4/6	67
$10^{-1.0}$	5/6	83
$10^{-1.5}$	4/6	67
$10^{-2.0}$	6/6	100
$10^{-2.5}$	0/6	0
IBV-RNA + RNase	0/6	0
IBV-RNA + DNase	6/6	100
HSB only	0/6	0

[a] RNA extracted from purified IBV particles (Materials and Methods) was diluted by serial half-log steps in high salt buffer (HSB) using a preparation with an initial concentration of 0.27 μg/0.2 ml. Confluent monolayers of CEF cells were incubated for 2 days at 37° following inoculation with 0.2-ml aliquots of diluted RNA. Thereafter, undiluted lysates obtained by freezing-thawing cells and fluids were inoculated into the allantois of embryonated eggs which were observed for mortality during a 7-day incubation at 37°. For testing the infectivity of viral RNA exposed to nucleases, IBV-RNA (1.2 μg) in low salt buffer was pretreated for 20 min with RNase or DNase as described in the footnote to Table 1.

[b] D/T = number of deaths/total number inoculated; most deaths occurred within 48 hr after inoculation.

[c] When titrated in eggs, the 48-hr harvest from CEF cultures receiving this input of IBV-RNA had an LD_{50} titer of $10^{4.2}$/ml.

this cell culture system for growth of the virus. Higher infectious virus yields and RNA infectivity end points might be obtained if deembryonated eggs were to be used for the primary RNA infections. We were unable to demonstrate RNA infectivity by direct inoculation into the allantoic sac of intact eggs, presumably owing to the degradative effects of nucleases present in the embryonic fluids. Although a systematic study was not conducted on facilitators of nucleic acid uptake, we can report that polycations such as DEAE-dextran and poly(L-ornithine) which were earlier shown to be active in other systems (Bishop and Koch, 1969) were less efficient than the hypertonic shock technique for promoting infection with IBV-RNA.

Polyadenylic Acid Content of IBV Genome RNA

The above findings prompted us to examine IBV viral RNA for the presence of poly(A) tracts using the methods of selective absorption on to cellulose nitrate filters (Lee *et al.*, 1972) and sedimentation analysis of adenosine-labeled viral RNA before and after exposure to mixtures of RNase A and T_1. All RNA extractions were performed using the SDS–phenol–chloroform technique, which was earlier shown to preserve the integrity of poly(A)-containing mRNA (Perry *et al.*, 1972).

The data presented in Table 4 illustrate that at least 20–30% of [^{3}H]uridine-labeled IBV viral RNA binds to cellulose nitrate filters or poly(U)-impregnated glass fibers. In contrast, ^{32}P-labeled genomic RNA of VSV and influenza virus exhibited no binding under identical conditions, an observation consistent with the fact that both VSV-RNA and WSN influenza RNA lack poly(A) tracts (Gillespie *et al.*, 1972; Roy *et al.*, 1973). We can also conclude that a portion of the IBV-RNA contains adenylate-rich sequences of at least 50–75 AMP residues, in view of the fact that the filter-binding assay cannot detect tracts below this range (Perry *et al.*, 1973). To demonstrate further that only a portion of virion RNA molecules contains poly(A), a preparation of ^{3}H-labeled IBV-RNA mixed with ^{32}P-labeled VSV-I RNA and VSV-III RNA was analyzed by sedimentation in a glycerol–SDS gradient and subsequent binding of gradient fractions to cellulose nitrate filters. These gradients were preferred for this purpose since they usually gave good resolution of RNA species. In agreement with the data of Table 4, approximately 25% of the total IBV-RNA population was found by this approach to contain poly(A)-rich regions (Fig. 5). No major differences appeared to exist between the size of RNA molecules which contain poly(A) and the size of those which do not. As expected, the VSV-I and VSV-III RNAs showed no binding to the cellulose nitrate filters in these experiments.

To determine the approximate size of the poly(A) sequences contained in IBV viral RNA molecules, virus grown in the presence of [^{3}H]adenosine was examined by sedimentation analysis following hy-

TABLE 4

ANALYSIS OF IBV-RNA FOR PRESENCE OF POLYADENYLIC ACID[a]

Experiment	Viral RNA	Acid-insoluble radioactivity (cpm)	Radioactivity bound (cpm)		Radioactivity bound (%)
			Poly(U)	Cellulose nitrate	
1	[^{3}H]IBV	8,556	1,957	–	23
	[^{32}P]WSN	1,066	52	–	4.8
2	[^{3}H]IBV	9,842	2,796	–	28.4
	[^{32}P]WSN	2,112	48	–	2.3
3	[^{3}H]IBV	17,201	–	3,416	20
4	[^{3}H]IBV	2,670	–	615	23
	[^{32}P]VSV	3,650	–	70	1.9

[a] ^{3}H-labeled RNA extracted from purified IBV particles was analyzed for poly(A) content by the cellulose membrane binding assay or poly(U) glass fiber binding assay described (Materials and Methods). In some experiments, ^{32}P-labeled WSN influenza RNA or VSV-RNA was added prior to filtration.

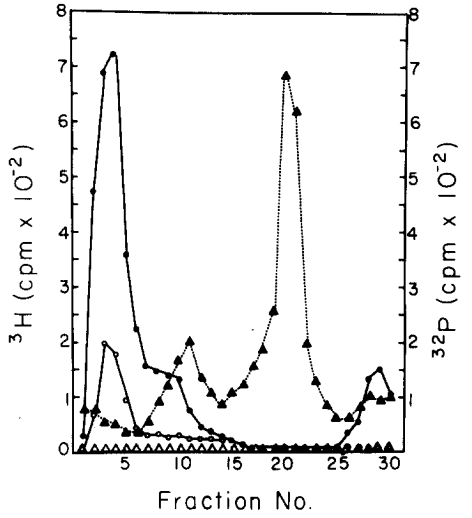

FIG. 5. Analysis of IBV-RNA by sedimentation and membrane binding. Tritium-labeled IBV-RNA was mixed with ^{32}P-labeled VSV-I RNA and VSV-III RNA and sedimented through 10–30% glycerol in STES run in a Spinco SW 27 rotor for 20 hr at 25,000 rpm (22°). One-half of each fraction was assayed for acid-insoluble radioactivity while the other half was first diluted in binding buffer and filtered through cellulose nitrate membranes to determine the amount of membrane-bound radioactivity. Acid-insoluble [^{3}H]IBV-RNA (closed circles), acid-insoluble [^{32}P]VSV-I RNA and [^{32}P]VSV-III RNA (closed triangles), membrane-bound [^{3}H]IBV-RNA (open circles), membrane-bound [^{32}P]VSV-RNAs (open triangles).

drolysis with RNase A and T_1 (see Materials and Methods). The total digestion products were sedimented in sucrose density gradients. The results obtained (Fig. 6) showed that the polyadenylated fraction consists of a heterogeneous population equal to or slightly larger than 4 S.

DISCUSSION

The *in vitro* cultivation technique employed in this study has permitted preparation of high-titered stocks of radiolabeled avian infectious bronchitis virus as a source of viral nucleic acid for biochemical analysis. We have also demonstrated the usefulness of this system for propagating and labeling other animal viruses which grow poorly in most tissue culture cell lines (Bishop *et al.*, 1972).

Our data support the conclusion that the genome of IBV consists of a single-stranded, covalently linked RNA species with a molecular weight of about 5.6×10^6. Thus, the IBV genome with a potential coding capacity for virus-specific proteins with a combined molecular weight of approximately 6×10^5 resembles that of paramyxoviruses such as Newcastle disease virus (Matthews, 1975). Beyond this, however, the similarity ends since IBV viral RNA is infectious and includes a population of RNA molecules containing tracts of polyadenylic acid. Whether only the polyadenylated species possesses infectivity has not been determined, although such experiments are feasible (Spector *et al.*, 1975). Enhancement of IBV-RNA infectivity per se in the presence of high salt concentrations may reflect, in part, a selective translational advantage of the viral RNA over cellular mRNA in light of recent reports that hypertonic shock results in a selective inhibition of host cell peptide chain initiation relative to that specified by various RNA viruses (Saborio *et al.*, 1974; Nuss *et al.*, 1975). Other substances such as calcium chloride or amphotericin

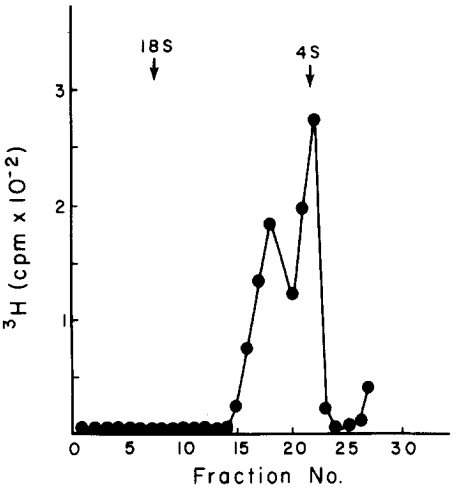

FIG. 6. Sedimentation analysis of the poly(A) contained in IBV-RNA. Viral RNA labeled with [^{3}H]adenosine was digested with pancreatic RNase (5 μg/ml) and RNase T_1 (40 units/ml) for 30 min at 37°. SDS was added at 0.05%, and the RNA was analyzed by velocity sedimentation through 15–30% sucrose in STES in an SW 27 rotor run at 25,000 rpm for 20 hr. ^{14}C-labeled guinea pig embryo cellular RNA was run simultaneously as a marker (arrows) in an identical gradient.

B which promote ingress of nucleic acids into eukaryotic cells have not been examined with IBV-RNA in our laboratory.

At variance with our data are the findings of two recent studies on the nature of the IBV genome. Tannock (1973) reported that particles of egg-grown avian bronchitis virus (Victoria S strain) contain heterogeneous size classes of discontinuous single-stranded RNAs ranging from 0.5×10^6 to greater than 3×10^6 daltons. In contrast, we have found IBV viral RNA to resolve as a large, homogeneous molecule by sedimentation analysis or electrophoretic separation in acrylamide gels. The most likely explanation for the findings obtained by Tannock is that late (48-hr) harvests of egg-grown IBV may contain defective virus with deleted genomes or may include virions with RNA which is prone to degradation during extraction. The differential lability of virion-associated RNA species from particles of oncornaviruses obtained at different times during infection illustrates the latter point (Bader and Steck, 1969; Cheung *et al.*, 1972). While Watkins and co-workers (1975) also suggest that the genome heterogeneity of IBV-RNA observed by Tannock may have resulted from degradative effects, these workers have reported the molecular weight of the viral RNA (Beaudette strain) as determined by PAGE analysis to be 9×10^6 despite their assignment of a sedimentation value of 50 S. The reason for this discrepancy is not clear, although RNA aggregation effects cannot be ruled out with certainty.

The composite properties of IBV viral RNA established in this investigation and the lack of detectable virion-associated transcriptase activity indicate that this animal virus contains a genome endowed with messenger function (Johnston and Bose, 1972). Thus, avian infectious bronchitis virus can be identified as a ribovirus of positive-strand genome polarity according to the currently accepted molecular convention (Baltimore, 1971). Future studies on the replication of avian infectious bronchitis virus in infected cells will be of particular interest to determine whether this coronavirus resembles other positive-strand enveloped viruses such as togaviruses (Fenner, 1976) in their molecular mode of biosynthesis. Our findings do not support the recent description of IBV as the type species of the family Coronaviridae as a virus containing a noninfectious RNA genome (9×10^6 daltons) and a virion-associated RNA polymerase (Tyrrell *et al.*, 1975). It would appear prudent, however, to exercise caution in suggesting that other members of the coronavirus group (Fenner, 1976) will be found to exhibit similar genome characteristics. This is especially true in view of the fact that initial grouping of these viruses has been largely based on common features of viral morphology and maturation rather than specific molecular or genetic attributes (McIntosh, 1974; Kapikian, 1975). As a further case in point, although picornaviruses, for example, as a class of positive-strand animal viruses contain an infectious RNA moiety, not all members of this group possess a polyadenylated genome (Porter *et al.*, 1974).

The present uncertainty surrounding the genomic properties of coronaviruses as a taxonomic group is further exemplified by the recent study of Garwes *et al.* (1975), in which it was suggested that transmissible gastroenteritis virus and hemagglutinating encephalomyelitis virus of swine contain 60–70 S RNA (9×10^6 daltons) which dissociates into 35 S subunits and 4 S RNA after heating. These presently classified coronaviruses of pigs thus appear to differ basically from IBV in their RNA structure, presuming that the preparations analyzed were free of an activated endogenous porcine oncornavirus (Lieber *et al.*, 1975). Until the nucleic acid components of other coronaviruses of diverse host origin are examined for their structural and biochemical properties, the taxonomic interrelationship of the viruses comprising this group will remain obscure.

ACKNOWLEDGMENTS

The authors are grateful for the expert technical assistance rendered by Connie Cannata, Christine Meyers, and Kathy Strezelec. This work was supported in part by Research Contract NIH-71-2077 of The Virus Cancer Program from the National Can-

cer Institute and Grant AI-09124 from the National Institute of Allergy and Infectious Diseases, USPHS. R. H. Stevens was supported in part as a predoctoral fellow under Training Grant CA 05234.

REFERENCES

BADER, J. P., and STECK, T. L. (1969). Analysis of the ribonucleic acid of murine leukemia virus. *J. Virol.* 4, 454-459.

BALTIMORE, D. (1971). Expression of animal virus genomes. *Bacteriol. Rev.* 35, 235-241.

BEAUDETTE, F. R., and HUDSON, C. B. (1937). Cultivation of the virus of infectious bronchitis. *J. Amer. Vet. Med. Assoc.* 90, 51-60.

BERNKOPF, H. (1949). Cultivation of influenza virus in the chorioallantoic membrane of de-embryonated eggs. *Proc. Soc. Exp. Biol. Med.* 72, 680-682.

BINGHAM, R. W. (1975). The polypeptide composition of avian infectious bronchitis virus. *Arch. Virol.* 49, 207-216.

BISHOP, D. H. L., and ROY, P. (1971). Kinetics of RNA synthesized by vesicular stomatitis virus particles. *J. Mol. Biol.* 57, 512-527.

BISHOP, D. H. L., ROY, P., BEAN, W. J., JR., and SIMPSON, R. W. (1972). Transcription of the influenza ribonucleic acid genome by a virion polymerase. III. Completeness of the transcription process. *J. Virol.* 10, 689-697.

BISHOP, J. M., and KOCH, G. (1969). Plaque assay for poliovirus and poliovirus specific RNAs. *In* "Fundamental Techniques in Virology" (K. Habel and N. Salzman, eds.), pp. 131-145. Academic Press, New York.

CHEUNG, K. R., SMITH, R. E., SMITH, M. P., and JOKLIK, W. K. (1972). Comparison of immature (rapid harvest) and mature Rous sarcoma virus particles. *Virology* 50, 851-864.

FENNER, F. (1976). The classification and nomenclature of viruses. Summary of results of meetings of the International Committee on Taxonomy of Viruses in Madrid, September, 1975. *Virology* 71, 371-378.

GARWES, D. J., POCOCK, D. H., and WIJASZKA, T. M. (1975). Identification of heat-dissociable RNA complexes in two porcine coronaviruses. *Nature (London)* 257, 508-510.

GILLESPIE, D., MARSHALL, S., and GALLO, R. C. (1972). RNA of RNA tumor viruses contains poly A. *Nature New Biol.* 236, 227-231.

HIERHOLZER, J. C., PALMER, E. L., WHITFIELD, S. G., KAYE, H. S., and DOWDLE, W. R. (1972). Protein composition of coronavirus OC 43. *Virology* 48, 516-527.

JOHNSTON, R. E., and BOSE, H. R. (1972). Correlation of messenger function with adenylate-rich segments in the genomes of single-stranded RNA viruses. *Proc. Nat. Acad. Sci. USA* 69, 1514-1516.

KAPIKIAN, A. Z. (1975). The coronaviruses. *Develop. Biol. Stand.* 28, 42-64.

LEE, S. Y., MENDECKI, J., and BRAWERMAN, G. (1971). A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. *Proc. Nat. Acad. Sci. USA* 68, 1331-1335.

LIEBER, M., SHERR, C. J., BENVENISTE, R. E., and TODARO, G. J. (1975). Biologic and immunologic properties of porcine type C viruses. *Virology* 66, 616-619.

MATTHEWS, R. E. F. (1975). A classification of virus groups based on the size of the particle in relation to genome size. *J. Gen. Virol.* 27, 135-149.

MCINTOSH, K. (1974). Coronaviruses: A comparative review. *Curr. Top. Microbiol. Immunol.* 63, 85-129.

NUSS, D. L., OPPERMANN, H., and KOCH, G. (1975). Selective blockage of initiation of host protein synthesis in RNA-virus-infected cells. *Proc. Nat. Acad. Sci. USA* 72, 1258-1262.

PERRY, R. P., LATORRE, J., KELLEY, D. E., and GREENBERG, J. R. (1972). On the lability of poly(A) sequences during the extraction of messenger RNA from polyribosomes. *Biochim. Biophys. Acta* 262, 220-226.

PERRY, R. P., GREENBERG, J. R., KELLEY, D. E., LATORRE, J., and SCHOCHETMAN, G. (1973). Messenger RNA: Its origin and fate in mammalian cells. *In* "Gene Expression and Its Regulation," p. 149. Plenum Press, New York.

PORTER, A., CAREY, N., and FELLNER, P. (1974). Presence of a large poly[rC] tract within the RNA of encephalomyocarditis virus. *Nature (London)* 248, 675.

REED, L. J., and MUENCH, H. (1938). A simple method of estimating fifty percent endpoints. *Amer. J. Hyg.* 27, 493-497.

ROY, P., REPIK, P., HEFTI, E., and BISHOP, D. H. L. (1973). Complementary RNA species isolated from vesicular stomatitis (HR strain) defective virions. *J. Virol.* 11, 915-925.

SABORIO, J. L., PONG, S. S., and KOCH, G. (1974). Selective inhibition of initiation of protein synthesis in mammalian cells. *J. Mol. Biol.* 85, 195-211.

SHELDON, R., JURAK, C., and KATES, J. (1972). Detection of polyadenylic acid sequences in viral and eukaryotic RNA. *Proc. Nat. Acad. Sci. USA* 69, 417-422.

SIMPSON, R. W., and GROUPÉ, V. (1959). Temperature of incubation as a critical factor in the behavior of avian bronchitis virus in chicken embryos. *Virology* 8, 456-469.

SIMPSON, R. W., and HIRST, G. K. (1961). Genetic recombination among influenza viruses. I. Cross reactivation of plaque-forming capacity as a method for selecting recombinants from the progeny of crosses between influenza A strains. *Virology* 15, 436-451.

SPECTOR, D. H., VILLA-KOMAROFF, L., and BALTI-

MORE, D. (1975). Studies on functions of polyadenylic acid on poliovirus RNA. *Cell* **6**, 41–44.

SPIRIN, A. S. (1962). Some problems concerning the macromolecular structure of ribonucleic acids. *Progr. Nucleic Acid Res. Mol. Biol.* **1**, 301–345.

TANNOCK, G. A. (1973). The nucleic acid of infectious bronchitis virus. *Arch. Gesamte Virusforch.* **43**, 259–271.

TYRRELL, D. A., ALMEIDA, J. D., BERRY, D. M., CUNNINGHAM, C. H., HAMRE, D., HOFSTAD, M. S., MALLUCCI, L., and MCINTOSH, K. (1968). Coronaviruses. *Nature (London)* **220**, 650.

TYRRELL, D. A., ALMEIDA, J. F., CUNNINGHAM, C. H., DOWDLE, W. R., HOFSTAD, M. S., MCINTOSH, K., TAJIMA, M., ZAKSTELSKAYA, L. YA., EASTERDAY, B. C., KAPIKIAN, A., and BINGHAM, R. W. (1975). Coronaviridae. *Intervirology* **5**, 76–82.

WATKINS, H., REEVE, P., and ALEXANDER, D. J. (1975). The ribonucleic acid of infectious bronchitis virus. *Arch. Virol.* **47**, 279–286.