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Coronavirus Multiplication Strategy

I. Identification and Characterization of Virus-Specified RNA

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We examined the synthesis of intracellular RNA in primary chicken embryo kidney cells infected with the avian coronavirus infectious bronchitis virus. Infected cells were labeled with $^{32}P_i$ in the presence of actinomycin D for the duration of the viral multiplication cycle, and nucleic acids were extracted, denatured, and analyzed on agarose slab gels. Six major RNA species were found. None of these RNAs was found in extracts of mock-infected cells. All six of the virus-specified RNAs (designated species A through F) were single stranded, and RNA species F had the same electrophoretic mobility as purified viral genome RNA. The molecular weights of the five subgenomic RNAs were estimated to be 0.8×10^6, 0.9×10^6, 1.3×10^6, 1.5×10^6, and 2.6×10^6 for species A through E, respectively. All of the RNAs were polyadenylated and are therefore likely to be viral mRNA's. The RNAs were synthesized in approximately constant proportions throughout the viral multiplication cycle. Intracellular RNA species A, B, C, D, and F and the purified viral genome were analyzed by RNase T_1 fingerprinting. The results confirmed the identification of RNA species F as the intracellular genome and the derivation of the four smaller RNAs from the genome. Fingerprinting also showed that the intracellular RNAs constitute a nested set such that the nucleotide sequence of each RNA is contained within all larger RNAs and each larger RNA contains an additional sequence congruent with its greater size. Finally, the possible modes of transcription and translation of the infectious bronchitis virus RNAs are discussed.

Coronaviruses, which are characterized by their surface "coronas" of widely spaced bulbous peplomers (20) have been isolated from a number of avian and mammalian species, including humans. Avian infectious bronchitis virus (IBV) is the prototype of the coronavirus family (26). The coronaviruses cause a wide range of diseases in different hosts, including acute respiratory pneumonitis and nephritis (IBV), hepatitis (murine hepatitis virus), gastroenteritis (porcine transmissible gastroenteritis virus), peritonitis (feline coronavirus), and a demyelinating disease of mice (JHM virus).

Recently, it has been established that the coronavirus genome is single-stranded, polyadenylated, nonsegmented RNA which is infectious (i.e., of positive polarity) (14, 15, 22) and that the virion does not contain a reverse transcriptase (20). Thus, the coronaviruses, together with the togaviruses and the picornaviruses, belong to class IV of the Baltimore scheme (1). Estimates of the molecular weight of the genome range from about 3×10^6 (25) to 9.0×10^6, with three reports giving values between 8×10^6 and 9×10^6 (15, 17, 27). If these latter estimates are correct, the coronavirus genome is the largest viral RNA genome known.

In comparison with the genome, there is a paucity of information regarding coronavirus proteins. Although there have been a number of studies on the structural proteins of the virion (16, 20), there is not yet a consensus on either the size or the number of these proteins. This may reflect the relative fragility of coronavirions to density gradient centrifugation. Intracellular viral proteins have been described in the murine system (2), but there is little information regarding their relatedness to the structural proteins. Furthermore, no identification has been made of any nonstructural protein, and therefore nothing is known about the protein composition of the virus-specified RNA-dependent RNA polymerase(s).

Our initial approach in attempting to elucidate the multiplication strategy of the coronaviruses has been to identify and characterize the species of intracellular viral RNA formed in chicken embryo kidney (CEK) cells infected with IBV. One previous report (21) suggested that cells infected with the murine coronaviruses JHM virus and A59 virus contain viral mRNA sedimenting between 10S and 28S. However, no evidence was presented that these were indeed viral species.

In this paper we demonstrate that chicken cells infected with IBV synthesize six discrete species of virus-specified RNA. These comprise the genome and five single-stranded RNAs, ranging in molecular weight from 0.8×10^6 to 2.6×10^6. All of these RNAs are polyadenylated. RNase T_1 fingerprinting of the four smallest RNAs established that these single-stranded RNAs are all subgenomic and therefore are probably all viral mRNA's with specific functions. Fingerprinting also revealed that these subgenomic RNAs form a nested set; that is, the sequence of each RNA is contained within the sequences of all larger RNA species. Finally, we discuss possible transcriptional and translational mechanisms for these overlapping RNAs.

MATERIALS AND METHODS

Materials. Actinomycin D and Sankyo RNase T_1 were purchased from Calbiochem-Behring Corp., agarose (type II) and hexadecyltrimethylammonium bromide were from Sigma Chemical Co., linear polyacrylamide (molecular weight, $>5 \times 10^6$) was from BDH, bisacrylylcystamine was from Pierce Chemical Co., proteinase K was from Merck & Co., Inc., and oligodeoxythymidylic acid [oligo(dT)]-cellulose (type 3) was from Collaborative Research Inc. Yeast tRNA (type X; Sigma) was purified by phenol-chloroform (1:1, vol/vol) extraction, precipitated with ethanol, recovered by centrifugation, dissolved in water to a concentration of 20 mg/ml, and stored at −20°C. Hydroxylapatite (HA; DNA grade; Bio-Rad Laboratories) was treated before use as follows; 20 g of HA was suspended in 150 ml of 0.5 M NaH_2PO_4 (pH 7.0) containing 0.1% diethylpyrocarbonate and boiled for 15 min. Fines were removed by aspiration and the HA was washed extensively with sterile water and TA buffer (10 mM Tris, 20 mM sodium acetate, 5 mM EDTA, pH 7.3) and stored as a concentrated slurry at 4°C. Carrier-free $^{32}P_i$ (285 Ci/mg) was supplied by ICN. Autoradiography was performed by using Kodak X-Omat R film. Cronex Lightning-plus intensifying screens were used to enhance sensitivity. Phenol, ether, and ethanol were redistilled before use. All other reagents were the highest grade commercially available.

Cells. CEK cells were prepared in the following manner. Kidneys were removed from 19-day-old White Leghorn chicken embryos and placed in a petri dish containing 0.9% NaCl. Kidneys were washed twice with 0.9% NaCl, transferred to a 10-ml syringe (without a needle), and expelled into a 100-ml trypsination flask (Bellco Glass, Inc.) containing 100 ml of 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. After 5 min at 37°C, the supernatant was discarded, and an equal volume of fresh trypsin solution was added. Incubation was then continued for 75 min at 37°C. The suspension was poured into two 50-ml centrifuge tubes (each containing 5 ml of heat-inactivated calf serum) and centrifuged at $200 \times g$ for 10 min at room temperature. The cell pellets were pooled, washed once by centrifugation in 0.9% NaCl, and suspended in medium 199 (Earle salts) supplemented with 5% heat-inactivated calf serum, 1% heat-inactivated chicken serum, 0.3% tryptose phosphate broth (Difco Laboratories), 10^4 U of penicillin per ml, 10 mg of streptomycin per ml, and 2,000 U of mycostatin per ml (growth medium). Typically, cells from 40 embryos were suspended in 250 ml of growth medium. Cells were plated out in plastic petri dishes (3×10^7 cells per 150-mm dish; 2.4×10^6 cells per 35-mm dish) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cultures were used 2 or 3 days after seeding. Monolayer cultures of HeLa cells in 32-ounce (960-ml) glass bottles were supplied by John Holland.

Viruses. The Beaudette strain (strain 42) of IBV was obtained from the American Type Culture Collection and after five passages in CEK cells was plaque purified (see below). The stocks used for the experiments described below were prepared from individual plaques by two to six low-multiplicity passages in CEK cells (see below). The Mahoney strain of type 1 poliovirus was obtained from John Holland. Our Semliki Forest virus stocks containing defective interfering (DI) particles (passage 7 or 8) were the stocks described previously by Bruton and Kennedy (3).

Passage of IBV in CEK cells. Cells in 150-mm petri dishes were washed with 0.9% NaCl and infected with virus diluted in 3% tryptose phosphate broth to give a multiplicity of infection of 0.05. After 90 min at 37°C, the inoculum was removed, and 25 ml of medium 199 containing 2% calf serum was added. The culture fluids were harvested after 24 h, fresh medium was added to the cells, and after an additional 24 h both harvests were clarified by centrifugation at $10,000 \times g$ for 25 min at 4°C and stored in aliquots at −70°C. This double harvest increased overall virus yield. The stocks from 24- and 48-h harvests were titrated and used interchangeably.

IBV plaque assay. Suspensions of IBV were serially diluted 10-fold in 3% tryptose phosphate broth. Cells in 35-mm petri dishes were washed once with 0.9% NaCl and infected with 0.2 ml of diluted virus. Two cultures were used for each dilution. After adsorption for 90 min at 37°C, the inoculum was removed, and the cells were overlaid with serum-free medium 199 containing 0.2% bovine serum albumin (Pentex fraction V; Miles Laboratories) in 0.9% agar (Noble agar [Difco]). Plaques were counted after 3 days at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air by removing the overlay and staining the cell sheet with crystal violet (9).

Radioactive labeling and extraction of intracellular RNA. CEK cells in 150-mm dishes were incubated in Glasgow modified minimal essential medium containing 1/10 the normal concentration of phosphate and 2% dialyzed calf serum (prelabeling medium) for 8 to 20 h before infection. The cells were then washed once with 0.9% NaCl, and 10 ml of virus stock was added to each plate (the multiplicity of infection varied from 10 to 120). After adsorption for 90 min at 37°C, the inoculum was replaced with 25 ml of phosphate-free Glasgow modified minimal essential medium containing 2% dialyzed calf serum and 1 μg of actinomycin D per ml (labeling medium). After an additional 1 h at 37°C the medium was replaced with fresh labeling medium containing 80 μCi of $^{32}P_i$ per ml

(25 ml/culture). At 13 h postinfection (defined relative to the end of the adsorption period), the cells were washed three times with ice-cold phosphate-buffered saline and once with 50 mM Tris (pH 7.4) containing 100 mM NaCl and 1 mM EDTA (TNE). Cytoplasmic nucleic acids were prepared by Triton N-101 lysis and phenol-chloroform-sodium dodecyl sulfate extraction, exactly as previously described (19).

Labeled poliovirus intracellular RNA was isolated from HeLa cells in an identical fashion, except that adsorption was for 15 min and the cytoplasm was harvested and extracted 6 h postinfection.

Semliki Forest virus intracellular standard and DI RNAs were labeled and extracted as described previously (19).

Preparation of IBV virion RNA. CEK cells in 150-mm dishes were incubated in prelabeling medium for 8 to 20 h before infection. The cells were washed once with 0.9% NaCl and infected with IBV in 3% tryptose phosphate broth to give a multiplicity of infection of 1.5. After adsorption for 90 min at 37°C, the inoculum was replaced with labeling medium (without actinomycin D) containing 50 μCi of $^{32}P_i$ per ml (20 ml/dish). At 18 h postinfection the culture fluid was harvested and clarified by centrifugation at 10,000 × *g* for 25 min at 4°C. Virus was purified from the supernatant by banding in 16-ml 20 to 55% (wt/vol) sucrose gradients prepared in TNE. Centrifugation at 4°C was for 17 h at 75,000 × *g* in an SW27 rotor. The virus from one 150-mm dish was placed onto a single gradient. Gradients were unloaded, and a sample of each fraction was Cerenkov counted. Peak fractions containing labeled virus (centered at a buoyant density of 1.18 g/ml) were pooled and diluted with cold TNE, and virus was pelleted by centrifugation at 65,000 × *g* for 2.5 h at 4°C in a type 30 rotor. Pellets were covered with 1.0 ml of TNE, left overnight at 4°C, and then gently resuspended, and the virion RNA was extracted immediately in one of the following ways.

(i) Extraction of total virion RNA for analytical gel electrophoresis. Resuspended virus was transferred to a 15-ml glass centrifuge tube, and 25 μg of tRNA was added. The volume was adjusted to 2.0 ml with TNE, 2% sodium dodecyl sulfate and 250 μg of proteinase K per ml were added, and the suspension was incubated at 50°C for 5 min and then at 20°C for 20 min. Finally, the RNA was extracted twice with phenol-chloroform and once with ether and then ethanol precipitated at −20°C (19).

(ii) Extraction and purification of virion RNA for fingerprinting. Purified virus was treated as described above, except that the initial suspension volume was 1 ml and a single phenol-chloroform extraction was used. The material was then divided into two aliquots, which were layered directly onto two 10.5-ml linear 15 to 30% (wt/vol) sucrose gradients prepared in 50 mM Tris (pH 7.4) containing 100 mM LiCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After centrifugation at 150,000 × *g* for 3 h at 10°C in an SW41 rotor, the gradients were fractionated, and a sample of each fraction was Cerenkov counted. Fractions containing full-length virion RNA (15) were pooled, 150 μg of tRNA was added, and the RNA was precipitated with 2.5 volumes of ethanol at −20°C.

Analytical agarose gel electrophoresis. Nucleic acids were recovered from alcohol by centrifugation, dried in vacuo over $CaCl_2$, denatured with glyoxal (18), and analyzed by electrophoresis on 1.2% horizontal agarose slab gels, as previously described (19).

Preparative acrylamide gel electrophoresis. The nucleic acids extracted from five 150-mm dishes were recovered from alcohol by centrifugation, dried in vacuo over $CaCl_2$, and dissolved in 200 μl of 0.1× TNE containing 5% sucrose and 0.002% bromophenol blue. This solution was then placed in the four slots (20 by 1.6 mm) of a 2% acrylamide–0.1% bisacrylamide or 3% acrylamide–0.2% bisacrylylcystamine vertical slab gel. The gel and electrode chamber buffer contained 26 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 8.0). The acrylamide-bisacrylamide gel was electrophoresed for 12 h at 60 V, and the acrylamide-bisacrylylcystamine gel was electrophoresed for 14 h at 100 V. Both gel systems were prerun for 1 h before application of the sample. Use of these acrylamide gel systems was necessary to obtain good separation of RNAs A through D for fingerprinting (see below).

Recovery of RNA from preparative gels. After electrophoresis RNA bands were located by autoradiography of the wet gels, excised, transferred to a petri dish, and washed three times with 20 ml of TA buffer. The gel slices were then transferred to wells cut in a precast horizontal 1.2% agarose gel in TA buffer and sealed into the wells with agarose (corresponding slices were pooled in common wells). A 3-mm-wide slot extending the length of each sample well was cut out of the agarose gel on the anode side of the sample well. This slot was filled with a slurry of HA in TA buffer (see above). During electrophoresis for 4 to 8 h at 100 V, the RNA migrated out of the gel slices and into the HA, where it bound (24). After electrophoresis the HA was transferred to a diethylpyrocarbonate-treated polypropylene chromatography column (Quick-sep; QS-Q; Isolab) fitted into a 15-ml polystyrene centrifuge tube. The column was then filled with TA buffer and centrifuged at 1,200 × *g* for 5 min at 20°C, and the eluate was discarded. A 1-ml amount of 400 mM NaH_2PO_4 (pH 7.0) was applied to the column, the HA matrix was resuspended, and the column was centrifuged as described above. The majority of the counts eluted in this phosphate wash. The column was then eluted with an additional 0.5 ml of the phosphate buffer, and the two phosphate eluates were pooled. The eluate was cooled to 4°C, 50 μg of tRNA was added and the RNA was precipitated by adding 0.1 ml of 0.1 M hexadecyltrimethylammonium bromide. After 30 min on ice, the suspension was transferred to two 1.5-ml polypropylene snap cap tubes and centrifuged for 5 min in an Eppendorf microcentrifuge. Without decanting, an additional 50 μg of tRNA was added to each tube, and the tubes were placed on ice for 30 min. After the tubes were centrifuged for an additional 5 min, the pellets were washed twice by centrifugation with 1.0 ml of cold TA buffer containing 50 μl of 0.1 M hexadecyltrimethylammonium bromide. Finally, the precipitates were dissolved in 1.5 ml of 50 mM Tris (pH 7.4) containing 1 mM EDTA and 1 M NaCl, and the RNA was precipitated with 2.5 volumes of ethanol at −20°C.

RNase T_1 fingerprinting. RNA was recovered

from alcohol by centrifugation, dissolved in 100 μl of water, and dried in vacuo over $CaCl_2$. Typically, 80,000 to 200,000 cpm of each RNA was fingerprinted. The RNA was digested with RNase T_1, and the resulting oligonucleotides were separated on two-dimensional slab gels as described previously (12), except that the second-dimension gel was 20% acrylamide–0.3% bisacrylamide and contained 0.5% linear polyacrylamide. These modifications made it possible to autoradiograph the gels at −70°C without cracking. Fingerprints were visualized by autoradiography for 2 days to 2 weeks at −70°C, using intensifying screens.

RESULTS

Kinetics of IBV growth in CEK cells. Before the intracellular synthesis of IBV-specified RNA was examined, it was essential to determine the one-step growth kinetics of our strain of IBV in CEK cells. Since preliminary experiments had shown that actinomycin D did not reduce the final yield of infectious virus from CEK cells by more than 1 $\log_{10}$ (11) and since we wished to use this drug in all of our labeling experiments, a one-step growth experiment (Fig. 1) was performed, in which 1.0 μg of actinomycin D per ml was added immediately after adsorption. After a latent period of about 4 h, there was a 4- to 5-h period of exponential virus production, after which virus formation declined. The final yield of virus in this experiment was 2×10^7 PFU/ml. In separate experiments (without actinomycin D) the final yield ranged from 2×10^7 to 4×10^8 PFU/ml. These yields correspond to a burst size of about 18 to 360 PFU/cell.

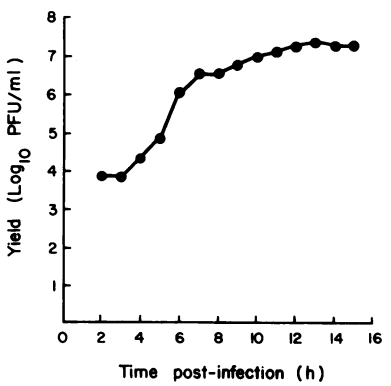

FIG. 1. *One-step growth curve of IBV in CEK cells. A single 150-mm petri dish culture of CEK cells was washed with 0.9% saline and infected with IBV at a multiplicity of infection of 20. After adsorption for 90 min at 37°C, the inoculum was replaced with 25 ml of labeling medium. After an additional 1 h the medium was replaced with 25 ml of fresh labeling medium, and at the times specified (relative to the end of the adsorption period) 200-μl samples were taken for plaque assay.*

These variations were probably due, at least in part, to the presence of varying numbers of nonpermissive fibroblasts in the monolayer cultures of permissive kidney cells. In view of the growth kinetics shown in Fig. 1, radiolabeling was performed during the time interval from 1 to 13 h postinfection.

Species of IBV-specified intracellular RNA. To examine IBV intracellular RNA synthesis, we labeled infected CEK cells with $^{32}P_i$ in the presence of actinomycin D, isolated cytoplasmic nucleic acids, and after glyoxal denaturation separated the nucleic acids by agarose gel electrophoresis (Fig. 2). Six major bands, which we designated A through F in order of increasing size, were reproducibly found in the extracts of infected cells (Fig. 2, lane b). None of these bands was found in mock-infected cell extracts (Fig. 2, lane a). The single high-molecular-weight band present in mock-infected cell extracts (Fig. 2, lane a, arrow) and the corresponding band found to varying extents in infected extracts was completely sensitive to DNase and insensitive to RNase and was therefore cellular DNA. By contrast, bands A to F were completely sensitive to RNase at both low (5 mM) and high (500 mM) NaCl concentrations and therefore are all single-stranded RNA (data not shown). The RNA extracted from purified virus particles (Fig. 2, lane c) comigrated with band F, strongly suggesting that this band was the intracellular form of the viral genome. In addition to these six major bands, we occasionally observed a number of minor bands (for example, see between bands B and C in Fig. 2, lane b, and Fig. 4, lane a). We have not yet attempted to characterize the minor bands and do not know whether they are of viral or cellular origin.

Molecular weights of intracellular RNAs. The molecular weights of IBV RNA species A to F were determined from the linear relationship between $\log_{10}$ molecular weight and electrophoretic mobilities of marker RNAs on agarose gels run under denaturing conditions (18). The marker RNAs were poliovirus RNA and several species of standard and DI Semliki Forest virus-specified RNA. This determination (Fig. 3) gave the following approximate values for the molecular weights of the IBV RNAs: species A, 0.8×10^6; species B, 0.9×10^6; species C, 1.3×10^6; species D, 1.5×10^6; species E, 2.6×10^6; and species F, 6.9×10^6. With the exception of species F, all of the IBV RNAs migrated within the limits of the calibration markers. Therefore, the molecular weight of species F may not be accurate.

Oligo(dT)-cellulose chromatography of intracellular RNAs. To determine the polarities of the IBV-specified RNAs, a labeled in-

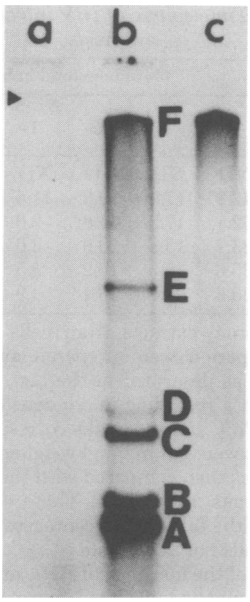

FIG. 2. *Species of RNA synthesized in CEK cells infected with IBV. Petri dish cultures of CEK cells were infected with IBV and labeled with $^{32}P_i$ from 1 to 13 h postinfection as described in the text. Parallel cultures were mock infected and labeled in the same way. The cultures were solubilized with Triton N-101, and nucleic acid extracts were prepared. In addition, two 150-mm petri dish cultures were labeled with $^{32}P_i$ for 18 h as described in the text for virion purification. Virus particles were purified from the culture fluids, and virion nucleic acids were extracted. The nucleic acid samples were denatured with glyoxal and electrophoresed on a 1.2% agarose slab gel as described in the text. The nucleic acids derived from approximately 10^6 cells of the mock-infected (lane a) and infected (lane b) cultures were analyzed. About 12,000 cpm of virus RNA (lane c) was applied to the gel. After electrophoresis the gel was fixed, dried, and autoradiographed for 30 h at −70°C. In this and all other RNA electrophoretograms migration was from top to bottom. The arrow in lane a indicates the position of cytoplasmic DNA.*

fected cell extract was chromatographed through oligo(dT)-cellulose (Fig. 4). A comparison of the gel profile of the extract before chromatography (Fig. 4, lane a) with the profile after chromatography (Fig. 4, lane b) clearly shows that RNA species A through F all bound to the matrix and therefore all contain a tract of polyadenylic acid. Thus, all of these species have positive polarity, and all are very likely to be viral mRNA's. Confirming evidence for the presence of a polyadenylic acid tract on each of the RNA species was obtained from RNase T_1 oligonucleotide fingerprinting (see below). The reduction in the general background (Fig. 4, lanes a and b) afforded by oligo(dT)-cellulose chromatography probably reflected removal of the non-polyadenylic acid-containing RNA fragments generated during the preparation of the nucleic acid extract.

Kinetics of synthesis of intracellular RNAs. In an attempt to determine whether the IBV-specified RNAs are all synthesized simultaneously throughout infection, we pulse-labeled parallel infected cultures for consecutive 2-h periods from 1 to 13 h postinfection. At the end of the pulse, cytoplasmic nucleic acid extracts were prepared, and the RNA species were separated by gel electrophoresis. From this analysis the relative amounts of RNA species A through F synthesized during each of the pulse periods were determined (Table 1). Two conclusions were drawn from this experiment. First, all of the RNAs were synthesized during the period from 3 to 13 h postinfection, and second, from 5 to 13 h the relative proportions of the RNA

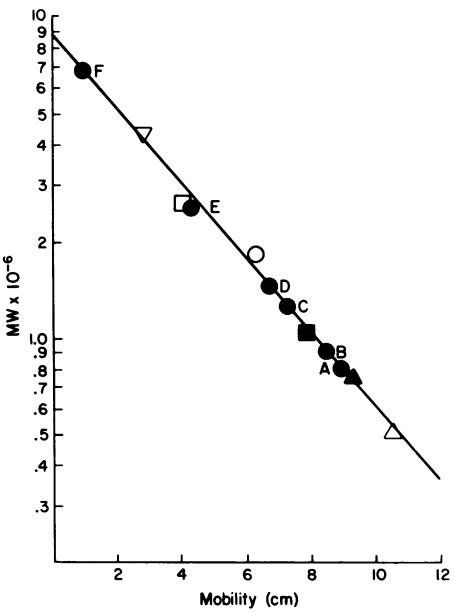

FIG. 3. *Determination of the molecular weights (MW) of the IBV intracellular RNAs. A labeled nucleic acid sample from IBV-infected CEK cells, which was prepared as described in the legend to Fig. 2, was denatured and electrophoresed on a 1.2% agarose slab gel. Marker labeled poliovirus RNA (□) (13) and a nucleic acid extract containing Semliki Forest virus standard virus 42S RNA (▽) and 26S RNA (○), together with Semliki Forest virus DI RNA species DIss X (△), DI ssD (▲), and DIssB (■) (23) (the molecular weight of DIss X is 0.5 × 10^6 [S.I.T. Kennedy, unpublished data]) were electrophoresed in parallel lanes. After electrophoresis the gel was fixed, dried, and autoradiographed, and the distance migrated by each RNA species (mobility) was measured with a ruler. The IBV RNA species are designated A through F.*

species were approximately constant. Thus, there appears to be little, if any, temporal regulation in the synthesis of the IBV-specified single-stranded RNAs. However, from 3 to 5 h postinfection the total incorporation of radioactivity into the RNAs was not high enough to permit accurate quantitation, and we must view with caution the results obtained for this pulse period.

Relative molarities of the intracellular RNAs. Table 2 shows a determination of the relative molarities of RNAs A through F, as estimated by determining peak areas from a densitometer tracing of a gel analysis of RNAs extracted from infected cells which had been labeled from 1 to 13 h postinfection. Table 2 shows the average results from two different infections. The molecular weights from Fig. 3 were used to calculate the molarities of RNA species A through E. A molecular weight of 8.1 $\times 10^6$ (15) was used for RNA species F (the genome). Table 2 shows the molarity of each RNA species relative to that of species A. It will be of interest to determine whether these molar RNA ratios are reflected in the relative amounts of their respective virus-specified polypeptide product(s).

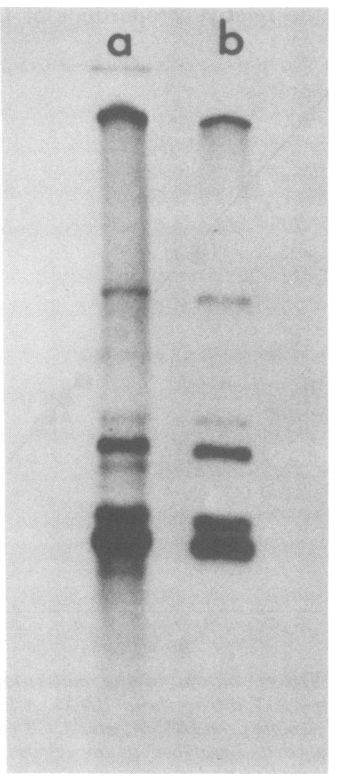

FIG. 4. *Polyadenylation status of the IBV intracellular RNAs. A labeled nucleic acid extract from IBV-infected CEK cells, which was prepared as described in the legend to Fig. 2, was denatured and electrophoresed on a 1.2% agarose slab gel (lane a). A second, identical portion of the extract was chromatographed over oligo(dT)-cellulose (7), and the polyadenylic acid-containing RNA was recovered and analyzed on an adjacent lane of the gel (lane b). Autoradiography was for 4 h with an intensifying screen at −70°C.*

TABLE 1. *Time course of IBV intracellular RNA synthesis*

Pulse period (h postinfection)	% of lane total in band:[a]					
	A	B	C	D	E	F (genome)
1–3	ND[b]	ND	ND	ND	ND	ND
3–5	42.9[c]	17.9[c]	9.2[c]	11.4[c]	3.5[c]	15.1[c]
5–7	32.1	17.2	15.8	2.0	7.0	26.0
7–9	33.7	13.8	20.9	3.0	4.9	23.8
9–11	35.6	15.6	16.2	5.5	5.5	21.7
11–13	33.6	24.6	16.4	3.9	6.1	15.4

[a] Cytoplasmic extracts from cells labeled during each pulse period were prepared and analyzed on agarose gels as described in the text. Each lane was scanned with a recording micro-densitometer (Joyce, Loebl, and Co.), and the peaks corresponding to each RNA species were cut out and weighed. The weight of each peak was then compared with the total weight of all of the peaks in the lane. These comparisons are expressed in the table as percentages.

[b] ND, Bands not detectable.

[c] Because of the low level of RNA at this early time, this value should be considered approximate (see text).

Oligonucleotide fingerprinting of the intracellular RNAs. To investigate possible sequence relationships among the IBV-specified RNAs and to establish their derivation from the genome, we digested RNA species A, B, C, D, and F with RNase T_1 and fingerprinted the resulting oligonucleotides by two-dimensional gel electrophoresis. In addition, we fingerprinted the genomic RNA from purified virus particles. The virion RNA fingerprint is shown in Fig. 5. Two features of this fingerprint are worthy of note. First, as evidenced by the large number of characteristic oligonucleotides (those above the position of the bromophenol blue tracker dye), the IBV genome is relatively complex. Second, although similar, the fingerprint shown in Fig. 5 is not identical to that published by Lomniczi and Kennedy (15). Thus, there has been some sequence divergence between the American Type Culture Collection variant of the Beaudette strain of IBV and the West German variant used by Lomniczi and Kennedy.

The fingerprints of RNA species A, B, C, D, and F are shown in Fig. 6. The polyadenylic acid tract of each RNA can be seen clearly in the top

TABLE 2. *Relative molarities of IBV intracellular RNAs*[a]

RNA species	Mol wt ($\times 10^{-6}$)	Molarity relative to species A
A	0.8	1.0
B	0.9	0.63
C	1.3	0.38
D	1.5	0.07
E	2.6	0.06
F	8.1	0.08

[a] The relative amount of each RNA species was determined as described in Table 1, footnote *a*. These amounts were then divided by the respective molecular weights and are expressed as fractions relative to the value for species A.

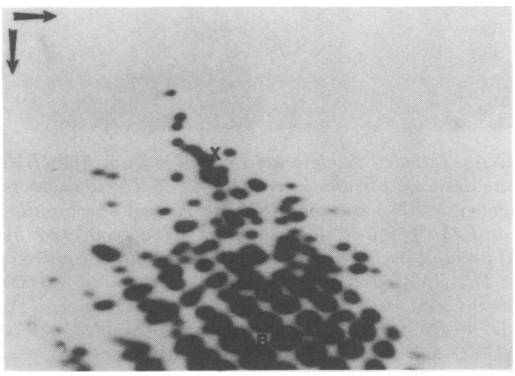

FIG. 5. *RNase T_1 fingerprint of IBV genomic RNA. Six 150-mm cultures of CEK cells were infected with IBV at a multiplicity of infection of 1.5 and labeled with $^{32}P_i$, and virus was purified as described in the text. Genomic RNA extracted with proteinase K-sodium dodecyl sulfate and then phenol-chloroform was gradient purified and ethanol precipitated. This RNA (100,000 cpm), together with 150 μg of carrier tRNA, was recovered and digested with RNase T_1, and the resulting oligonucleotides were fractionated by two-dimensional polyacrylamide gel electrophoresis as described in the text. In this and all subsequent fingerprints electrophoresis in the first dimension was from left to right and in the second dimension was from top to bottom. Autoradiography was for 2 weeks at −70°C with an intensifying screen. X and B indicate the positions of xylene cyanol FF and bromophenol blue, respectively.*

left corner of each fingerprint (12). Three important conclusions were drawn from these fingerprints. First, the fingerprint of RNA species F (Fig. 6e.) is indistinguishable from that of the genome (Fig. 5). This establishes the identity of species F as the intracellular form of the virion RNA. Second, it is clear that all of the oligonucleotide spots from RNA species A through D are contained within the fingerprint of the genome. Thus, these species are all subgenomic RNAs. Third, with one exception (Fig. 6a, spot 1), all of the oligonucleotide spots characteristic of species A are found in species B. Spot 1 is present in species A, absent from species B, but present in all of the other fingerprints. In addition, species B contains six unique spots not found in species (Fig. 6b, arrowheads). Similarly, all of the spots characteristic of species B are found in species C, and species C contains 11 additional unique spots (Fig. 6c, arrowheads). Likewise, all of the oligonucleotide spots characteristic of species C are found in species D, and species D contains one additional unique spot (Fig. 6d, arrowhead). A single spot (Fig. 6c, spot 2) is present in species C but absent from species D. This spot was also found in the fingerprint of the genomic RNA. The numbers of additional unique spots which species B has relative to species A, species C has relative to species B, and species D has relative to species C approximately correspond to the respective increments in molecular weights. We interpret these overlapping fingerprint patterns as showing that the nucleotide sequence of species A is contained within species B, that the sequence of B is contained within species C, and that the sequence of species C is contained within species D. Thus, the RNAs form a nested set, with each larger species containing an additional nucleotide sequence.

Are the intracellular RNA species DI RNAs? In view of the observation that almost every group of animal viruses can spawn DI particles containing truncated forms of the standard virion genome, we felt it important to evaluate the possibility that one or more of the IBV subgenomic RNAs might be DI. At the beginning we considered this possibility unlikely, because all of the virus stocks employed in the experiments described here were prepared by low-multiplicity passage (multiplicity of infection, less than 0.05). Nevertheless, if DI particles were present at any passage, then additional passaging would be expected to change the amount of intracellular DI genomic RNA; that is, the proportions of RNA species A through F would change. This did not occur: the relative proportions of the intracellular RNA species were independent of the passage number of the inoculum (Fig. 7A) over six passages.

As a final test for the absence of DI particles, the following experiment was performed. Virus labeled with $^{32}P_i$ was purified and divided into two portions. One portion was directly extracted with phenol-chloroform, and the viral RNA was denatured and electrophoresed on an agarose gel. Only a single sharp RNA band (Fig. 7B) was observed between the origin and the tracker dye front. Thus, the virus present in this purified

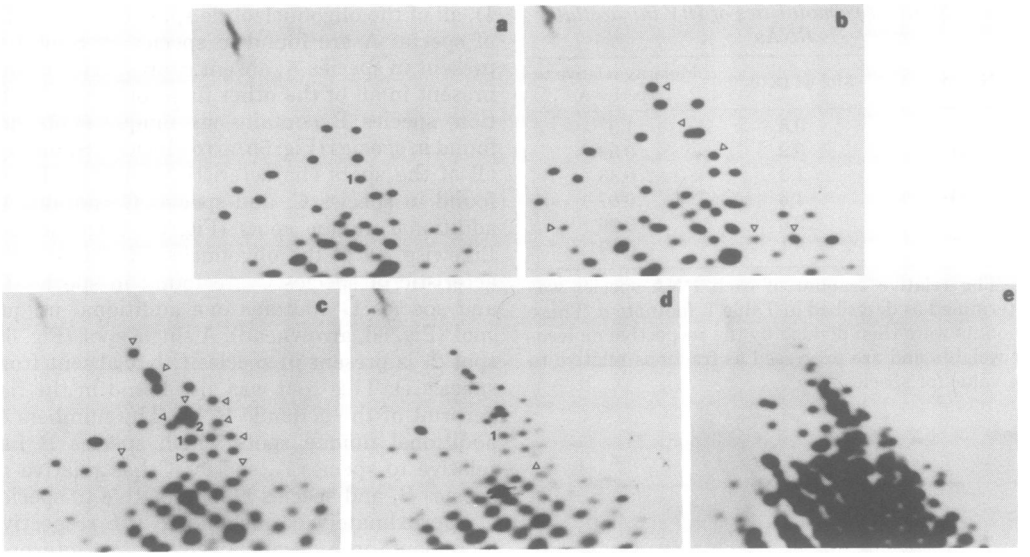

FIG. 6. *RNase T_1 fingerprints of IBV intracellular RNAs. Labeled nucleic acid extracts from five IBV-infected 150-mm petri dish CEK cultures were prepared as described in the legend to Fig. 2. These extracts were then electrophoresed on acrylamide-bisacrylamide gels (for RNA species A, D, and F) and acrylamide-bisacrylylcystamine gels (for RNA species B and C), and the resolved RNA species were recovered by adsorption to and elution from HA (see text) and alcohol precipitated. Each species (together with approximately 100 μg of tRNA) was recovered and fingerprinted. The RNA species contained from 80,000 to 200,000 cpm, and autoradiography at −70°C with intensifying screens was for 2 days to 2 weeks. (a) RNA species A. (b) RNA species B. (c) RNA species C. (d) RNA species D. (e) RNA species F. The significance of the spots marked with numbers and arrowheads is given in the text.*

preparation contained only a single species of RNA, the standard virus genome. The second portion was used to infect CEK cells directly, which were then labeled from 1 to 13 h postinfection in the presence of actinomycin D, and cytoplasmic nucleic acids were extracted. This extract was then analyzed by gel electrophoresis (Fig. 7B). A comparison of this profile with the profile of an extract from cells infected with unpurified (passage 2) virus (Fig. 7B, lane c) showed that the RNAs specified by purified virus were present in the same relative proportions as those specified by passage 2 virus. These observations demonstrate that cells infected with virus particles demonstrably free of any RNA species other than the standard virus genome synthesize all of the subgenomic RNAs in normal proportions.

Together, these observations strongly indicate that the subgenomic RNAs described in this paper are not DI RNAs but rather RNAs characteristic of the multiplication of standard virus particles. However, because no description of any coronavirus DI RNA exists, we cannot rigorously exclude the possibility that one or more of the subgenomic RNA species are indeed DI. Recent observations, however, show that on high-multiplicity serial passaging, particles accumulate which specify an RNA species migrating between RNA species E and F (R. Franco, J. Mercer, D. Stern, and S. I. T. Kennedy, unpublished data). This RNA species is therefore a likely candidate for a DI RNA species.

DISCUSSION

The results reported above establish that IBV directs the synthesis of five intracellular subgenomic RNAs (species A, B, C, D, and E), together with the intracellular genome (species F). As evidenced by oligo(dT)-cellulose chromatography and RNase T_1 oligonucleotide fingerprinting, all of these RNAs are polyadenylated and are therefore probably viral mRNA's. All six intracellular RNAs are synthesized in roughly constant proportions throughout the multiplication cycle, and serial passaging does not change these proportions significantly. A similar spectrum of RNAs has been observed recently in cells infected with murine coronavirus A59 (J. Leibowitz, personal communication).

As estimated here, the molecular weight of the viral genome is 6.9×10^6. Because this value was obtained by gel electrophoresis and involved extrapolation of the calibration curve, we consider it to be less accurate than the molecular weight value of 8.1×10^6, which was determined

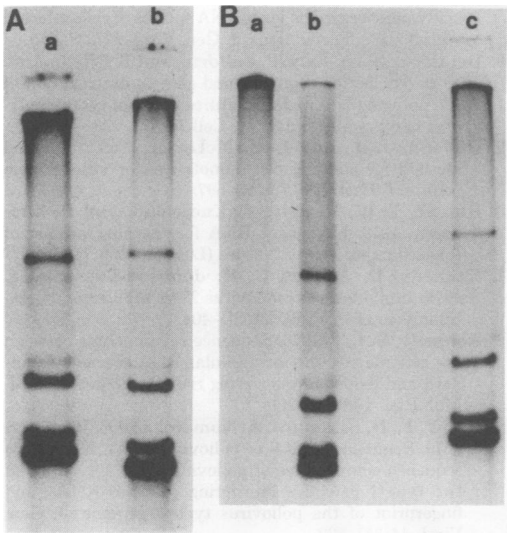

FIG. 7. *Possible IBV DI RNA species. (A) Labeled nucleic acid extracts from CEK cells infected with passage 2 (lane a) and passage 6 (lane b) virus were prepared as described in the legend in Fig. 2, denatured, and analyzed on two 1.2% agarose slab gels. Equal amounts of the two extracts were analyzed. (B) The unfractionated RNAs isolated from purified virus (lane a) and from cells infected with this virus (lane b) and unpurified passage 2 virus (lane c) were prepared, denatured, and analyzed on 1.2% agarose gels. Equal amounts of the two cell extracts were analyzed.*

previously by complexity measurements. However, the genome fingerprint in this paper differs somewhat from the one published previously (15). The two virus stocks were obtained from different sources and must therefore have diverged during passaging. Unfortunately, no detailed passage history exists for either of the two stocks, and so we cannot ascertain how many times they have been passaged independently or what previous cell systems have been employed. It will be of interest to determine to what extent the two strains cross-react serologically.

We fingerprinted subgenomic RNA species A through D. With the exception of two oligonucleotides (spots 1 and 2), the spots found in each RNA are also found in all larger members of the group and each successively larger RNA contains additional spots congruent with its greater size. The subgenomic RNAs therefore form a nested set. We have clear evidence that RNA species E also belongs to this set (Stern and Kennedy, manuscript in preparation). At the present time we are unable to fit the two anomalous spots into any tenable sequence pattern. The two spots are immediately adjacent to one another in the fingerprints and may therefore be structurally related. For example, they may be methylated homologs of one another or 5'-terminal cap oligonucleotide variants. Direct sequencing should reveal their relationship to one another and might afford an explanation for their electrophoretic behavior.

Since all of the RNAs contain a tract of polyadenylic acid, presumably at their 3' termini, then the molecular weight increments in going from species A to species D probably represent 5'-terminal sequence extensions. Although this is not the only possibility (for example, the additional sequences could be 3' terminal), we consider it to be the most likely possibility, if only by analogy with other virus systems. For example, the alphaviruses and tobacco mosaic virus both direct the synthesis of subgenomic mRNA's (5, 10). In both of these cases the nucleotide sequences of the subgenomic species are located inward from the 3' end of the genome. Moreover, polyoma virus directs the synthesis of two mRNA's (coding for VP2 and VP3), the smaller of which represents the 3'-terminal sequence of the larger (8). All of these systems illustrate a common viral solution to the apparent inability of eucaryotic protein-synthesizing apparatuses to initiate translation internally. Thus, by synthesizing 3'-terminal overlapping RNAs, cryptic initiation sites present in larger RNAs become exposed at or near new 5' termini. Currently, we are determining the sequence relationships between the subgenomic RNAs and the viral genome by determining the 5'-to-3' order of the genomic RNase T_1 oligonucleotides. Preliminary observations indicate that the subgenomic RNAs are indeed all located inward from the 3' end of the genome.

Two schemes have been described for the translation of overlapping RNAs. For the alphaviruses and tobacco mosaic virus, the larger (genomic) RNA is translated into polypeptides which do not overlap with the translation products of the respective subgenomic mRNA's (4, 6, 10). For polyoma virus mRNA's the translation product of the smaller RNA (VP3) is contained within that of the larger RNA species (VP2). Thus, in this case not only the RNAs but also the polypeptide products overlap (8). For IBV both schemes must therefore be considered although with five subgenomic RNAs some combination of both schemes could be present. In addition, it is possible that the RNAs could be translated in different reading frames. Of these several possibilities we have preliminary evidence in favor of the nonoverlapping translation scheme. This scheme predicts translation of the subgenomic RNAs to give polypeptides ranging in molecular weight from 10,000 to 110,000. These values are in good agreement with our

estimate (L. Burgess, S. Linesch, and S. I. T. Kennedy, manuscript in preparation) and the estimates of others (16) for the molecular weight size ranges of IBV structural proteins. Currently, we are studying the in vitro translation of the subgenomic RNAs, together with the translation of virion RNA. Since this latter RNA is infectious, it must encode not only the structural proteins but also the nonstructural proteins, including the viral RNA-dependent RNA polymerase(s).

Finally, it is pertinent to consider the possible mechanism(s) of synthesis of the subgenomic RNAs. At least three possible schemes can be envisioned. In the first all of the subgenomic RNAs would be transcribed individually from a negative strand identical in size to the genomic RNA. This would involve either internal transcriptive initiation or termination or both. This strategy (internal transcriptive initiation) is employed in the synthesis of the alphavirus subgenomic RNA (5). Alternatively, each of the IBV RNAs could be transcribed from a negative strand template identical in size to its respective progeny RNA. In this case the subgenomic negative strand templates would presumably be transcribed, at least initially, from genomic RNA. A third possibility is that the subgenomic RNAs all originate by nucleolytic processing of full-length genomic RNA. An analysis of the replicative form(s) and the replicative intermediate(s), together with a determination of the UV target size of the template(s), should distinguish among these possibilities.

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