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Genome of Infectious Bronchitis Virus

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Techniques are described for the growth and rapid purification of the avian coronavirus infectious bronchitis virus (IBV). Purified IBV has a sedimentation coefficient of 320S and a buoyant density of 1.22 g/ml in sucrose-deuterium oxide equilibrium gradients. IBV RNA extracted by proteinase K in the presence of sodium dodecyl sulfate and further purified by phenol extraction and gradient centrifugation is single stranded and has a sedimentation coefficient of 64S, as determined by isokinetic gradient centrifugation. Analysis on sucrose gradients under both aqueous and denaturing conditions together with agarose gel electrophoresis in the presence of the chaotropic agent methylmercuric hydroxide gave a value of 8×10^6 for the molecular weight of IBV RNA. This value was confirmed by RNase T_1 fingerprinting, which also indicated that IBV RNA is haploid. No evidence was found of subunit structure in IBV RNA. From these results together with the recently reported observation that IBV RNA is infectious and contains a tract of polyadenylic acid (Lomniczi, J. Gen. Virol., in press), we conclude that the genome of the coronaviruses is a single continuous chain of about 23,000 mononucleotides that is of messenger polarity.

Infectious bronchitis virus (IBV) is a member of the coronavirus group of animal viruses (20). Members of this group infect such disparate hosts as pigs, mice, fowl, and humans. In adult chickens, infection with IBV causes severe impairment of egg laying and results in respiratory signs in young chicks (6).

Studies on the structure of IBV have shown that the virus particle contains a lipoprotein envelope surrounding an electron-dense nucleocapsid. The external proteins of this envelope are arranged as a characteristic corona of spike projections (20).

Although it is clear that the genetic material of coronaviruses is single-stranded RNA, its structure and ploidy are uncertain. On the one hand, it has been reported that the genome is a single continuous strand with a molecular weight of about 9×10^6 (27); on the other hand, dissociation studies have led to the conclusion that the RNA consists of several fragments with an organization analogous to that of the oncoronaviral genome (9). The recent observations that IBV RNA is infectious and contains a tract of polyadenylic acid [poly(A)] clearly establish that the genome is of positive polarity (Lomniczi, J. Gen. Virol., in press).

In the present paper we report on the size, organization, and genetic content of IBV RNA. We show, by aqueous isokinetic gradient analysis, that the genome has a sedimentation coefficient of 64S. By use of denaturing conditions in both sucrose gradients and agarose gels, we estimate the molecular weight of IBV RNA to be $8.1 \pm 0.2 \times 10^6$. RNase T_1 oligonucleotide fingerprinting confirmed this molecular weight value and also showed that the genome is largely, if not entirely, haploid.

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MATERIALS AND METHODS

Materials. Proteinase K was obtained from Boehringer gmbH, Mannheim, West Germany, and formamide was obtained from Hopkin and Williams Ltd., London, England. Sodium dodecyl sulfate (SDS) was especially pure grade from British Drug Houses, Poole, England, and sucrose was RNase-free ultrapure from Schwarz/Mann, Orangeburg, N.Y. [5-^{3}H]uridine (28 Ci/mmol), [^{14}C]uridine (55 Ci/mmol), and [^{32}P]orthophosphate (94 Ci of phosphorus per mg) were supplied by the Radiochemical Centre, Amersham, England. RNase T_1 (a Sankyo product) was obtained from Calbiochem, London.

Viruses and cells. The Beaudette strain of IBV was obtained from V. von Bülow, Tübingen, West Germany, and used throughout. The origin of the Sindbis virus and L strain of Newcastle disease virus (NDV) has been described (18). Poliovirus and Sendai virus were obtained from the National Institute of Medical Research, London, and plaque purified twice before use. Semliki Forest virus was the *ts*$^+$ strain described by Kennedy and Burke (14). IBV was grown in primary chicken embryo kidney cells prepared from 17-day-old embryos (10). The cells

were cultivated in medium 199 containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.2, and 8% calf serum (growth medium). Chicken embryo fibroblast cultures were prepared as described previously (22) and used for the growth of Sindbis virus, Semliki Forest virus, Sendai virus, and NDV. Poliovirus was grown in HeLa cells cultivated in Glasgow-modified minimal essential medium containing 7.5% heat-inactivated fetal calf serum.

Labeling of viral RNA. Chicken embryo kidney cultures in 140-mm plastic petri dishes were infected with IBV at a multiplicity of about 1. After adsorption for 1 h at 37°C, the inoculum was replaced with medium 199 containing 1% calf serum and 30 μCi of [^{3}H]uridine per ml or 50 μCi of [^{32}P]orthophosphate per ml. In the latter case, phosphate-free medium containing 1% dialyzed calf serum was used. Virus was harvested at 16 h after infection. Radioactive Sindbis virus, Semliki Forest virus, NDV, and Sendai virus were prepared in an identical fashion but with chicken embryo fibroblasts. Radioactive poliovirus was prepared by growth in HeLa cells as described previously (5).

Preparation of labeled rRNA's. Chicken fibroblasts were labeled with [^{14}C]uridine (2 μCi/petri dish culture) for 18 h, and the rRNA's were extracted as previously described (21).

Virus purification. All procedures were carried out at 0 to 4°C. Tissue culture fluid containing labeled IBV was clarified by centrifuging at 10,000 × *g* for 30 min, and virus was pelleted from the supernatant by centrifugation in a 3 × 70 ml rotor (MSE 65 ultracentrifuge) for 2 h at 65,000 × *g* through a 30% (wt/vol) sucrose cushion prepared in NTE buffer (100 mM NaCl-10 mM Tris-1 mM EDTA, pH 7.0). The pellet was gently suspended in NTE, and the resulting suspension was clarified by centrifugation at 3,000 × *g* for 15 min. Sindbis virus and NDV were purified by an identical procedure. Sendai virus, Semliki Forest virus, and poliovirus were purified as described previously (12).

Sedimentation analysis of IBV. Velocity sedimentation was performed by using 12 ml of 10 to 40% (wt/vol) linear sucrose gradients centrifuged at 110,000 × *g* for 1.5 h or 12 ml of isokinetic sucrose gradients with a top concentration of 15% (wt/vol) sucrose (23). Equilibrium sedimentation was for 15 h at 120,000 × *g*, using 12 ml of 10 to 55% (wt/vol) linear sucrose gradients prepared in 98% deuterium oxide (7). Sedimentation analyses were performed in a 6 × 14 ml titanium rotor.

Isolation of viral RNA. Suspensions of purified IBV at a concentration of 1 to 2 mg of protein per ml were treated with 1% SDS and incubated with 300 μg of proteinase K per ml (11, 17) at 50°C for 5 min and then at 25°C for 30 min. 2-Mercaptoethanol was added to 0.1%, and the RNA was extracted three times with redistilled phenol saturated with 5 mM EDTA, pH 5.0. The final aqueous phase was made 0.2 M in sodium acetate, pH 5.0, and the RNA was precipitated at −20°C after the addition of 2.5 volumes of redistilled ethanol. The RNA was recovered by centrifugation at 5,000 × *g* for 10 min at 4°C, washed several times with 70% ethanol, dried in a stream of N_2, dissolved in a small volume of LES buffer (1 mM LiCl-5 mM EDTA-0.25% SDS, pH 5.0), and further purified by centrifugation at 200,000 × *g* for 4 h at 10°C on 12-ml 15 to 30% (wt/vol) linear sucrose gradients prepared in 10 mM Tris, pH 7.0, containing 100 mM LiCl-1 mM EDTA-0.25% SDS. After centrifugation, the gradient was unloaded to give about 20 fractions, and the radioactivity in a 30-μl sample of each fraction was determined. Fractions from the bottom quarter of the tube containing peak radioactivity (see Fig. 2a) were pooled, and the RNA was precipitated with 2.5 volumes of ethanol at −20°C. Sindbis virus and NDV RNAs were prepared in an identical fashion. Semliki Forest virus, Sendai virus, and poliovirus RNAs were isolated by use of the phenol-chloroform-2-mercaptoethanol-SDS procedure described previously (21).

Sedimentation analysis of IBV RNA. The sedimentation coefficient of IBV RNA was determined on 12-ml isokinetic sucrose gradients with a top concentration of 15% (wt/vol) sucrose containing 0.25% SDS (23). Centrifugation was at 5°C for 5.5 h at 200,000 × *g*. IBV RNA was also analyzed under denaturing conditions on 12-ml 5 to 15% (wt/vol) linear sucrose gradients prepared in 70% deionized formamide in 10 mM Tris, pH 7.0, containing 1 mM LiCl, 1 mM EDTA, and 0.25% SDS. RNA samples were dissolved in 70% formamide in LES buffer, incubated at 25°C for 30 min, and then centrifuged at 200,000 × *g* for 5 h at 12°C.

Agarose gel electrophoresis. One percent agarose slab gels (18 by 14 by 0.1 cm) containing 4 mM methylmercuric hydroxide were cast as previously described (1). The gel and reservoir buffer was 0.05 M sodium borate plus 0.01 M sodium sulfate, pH 8.1, containing 1 mM EDTA (E buffer). ^{32}P-labeled RNA samples were recovered from alcohol by centrifugation, dried in a stream of N_2, and dissolved in 0.1 E buffer containing 10% glycerol and 4 mM methylmercuric hydroxide. Electrophoresis was at an 80-V fixed potential for 16 h. After electrophoresis, gels were fixed in methanol, dried at 37°C under vacuum, and autoradiographed on Kodirex X-ray film.

Oligonucleotide fingerprinting. Approximately 35 μg of ^{32}P-labeled IBV RNA (specific activity, 0.74 × 10^6 cpm/μg) and 20 μg of ^{32}P-labeled Semliki Forest virus RNA (specific activity, 1.1 × 10^6 cpm/μg), each containing 100 μg of carrier tRNA, were recovered from alcohol by centrifugation, dried in a stream of N_2, dissolved in 15 μl of 10 mM Tris, pH 7.6, containing 10 μg of RNase T_1, and digested for 30 min at 37°C. Fractionation of the T_1 oligonucleotides was performed by two-dimensional polyacrylamide gel electrophoresis as described previously (13). After autoradiography for 43 h, selected oligonucleotides were recovered, counted by Cerenkov radiation, and digested with pancreatic RNase (100 μg/ml for 30 min at 37°C), and their products were analyzed as previously described (3).

RESULTS

Sedimentation coefficient and buoyant density of IBV. Preparations of IBV, purified as described above, were analyzed by velocity

(Fig. 1b) and equilibrium (Fig. 1a) centrifugation. With Sindbis virus (sedimentation coefficient, 225S) as the marker (26), the sedimentation coefficient of IBV was estimated to be 320S. Analysis on sucrose-D_2O equilibrium gradients gave a single coincident peak of infectivity and radioactivity at a buoyant density of 1.22 g/ml. This value is similar to that of other enveloped viruses, such as NDV (7).

Strandedness of IBV RNA. During preliminary experiments it became clear that to prepare IBV RNA with reproducible hydrodynamic and electrophoretic properties, both the method of virus purification and the RNA extraction procedure had to be considered. The technique finally adopted and detailed above involved RNA extraction from partially but rapidly purified virus by using proteinase K followed by gradient centrifugation. IBV RNA prepared in this way gave a single sharp peak on a linear sucrose velocity gradient (Fig. 2a). The strandedness of IBV RNA was determined by RNase digestion and examination of the sedimentation properties of the RNA under varying salt conditions (8, 26). In the first of these approaches, portions of each fraction from the sucrose velocity gradient shown in Fig. 2a were digested with pancreatic RNase (20 μg/ml for 20 min at 25°C), trichloroacetic acid precipitated, and counted (21). As Fig. 2a shows, this treatment completely digested IBV RNA. In the second approach, IBV RNA was analyzed by sucrose velocity centrifugation in buffer containing either 100 or 1 mM LiCl (Fig. 2b). Relative to rRNA's centrifuged in the same tubes, the sedimentation coefficient of IBV RNA decreased from about 60S in 100 mM LiCl to 50S in 1 mM LiCl. Recovery of the 50S material and recentrifugation on a gradient containing 100 mM LiCl gave an identical sedimentation profile to that obtained with native IBV RNA, confirming that the change in sedimentation behavior was due to a reversible conformational alteration. Taken together, these results confirm the observation of Watkins et al. (27), that IBV RNA is single stranded.

Sedimentation coefficient and molecular weight of IBV RNA determined under nondenaturing conditions. When isokinetic sucrose gradient centrifugation (23) in the presence of SDS was used, the sedimentation coefficient of IBV RNA was found to be 64S. Values of 51S and 47S were found for NDV and Sindbis virus RNA, respectively (Fig. 3a). These values closely compare not only with published estimates (17, 24), but also with the values obtained by band sedimentation in a model E ultracentrifuge (Kennedy, unpublished data). A plot of the logarithm of the molecular weight of Sindbis virus RNA (4.2×10^6 [24]), of NDV RNA (5.6×10^6 [4, 16]), and of 28S and 18S rRNA's (15) against the logarithm of distance sedimented from the meniscus gave an approximate straight line (Fig. 3b) with a molecular weight of about 8×10^6 for IBV RNA.

Sedimentation properties and molecular weight determination of IBV RNA under denaturing conditions. The sedimentation behav-

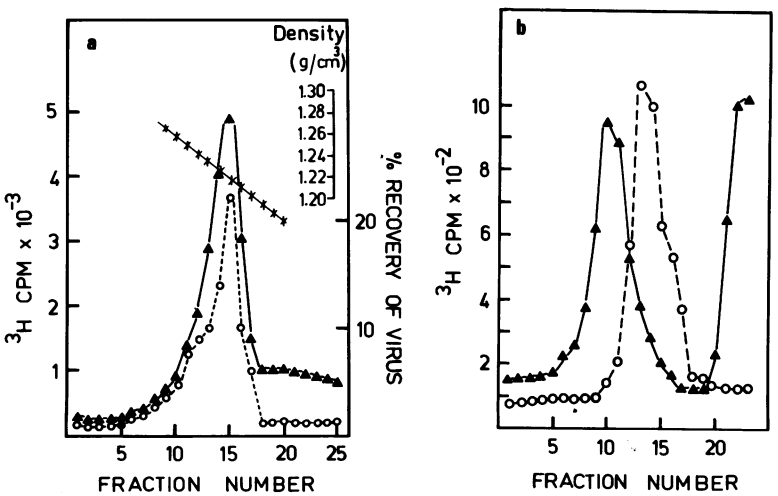

FIG. 1. *Equilibrium and velocity centrifugation of IBV. (a) A 0.2-ml amount of virus was centrifuged on 12 ml of a 98% D_2O–10 to 55% sucrose (wt/vol) gradient at 120,000 × g for 15 h at 4°C. The radioactivity (▲) of 40-μl samples and the infectivity (○) were determined as described in the text. (b) IBV (▲) and Sindbis virus (○) were centrifuged on a 12-ml isokinetic sucrose gradient with a top concentration of 15% (wt/vol), prepared by the method of Noll (23). Centrifugation was performed at 2°C for 1.5 h at 110,000 × g. The radioactivity was determined in 20-μl samples.*

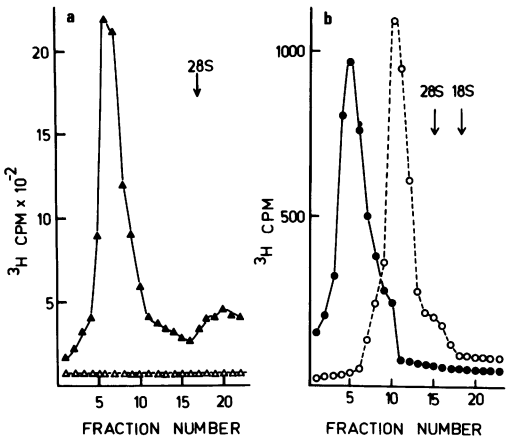

FIG. 2. *Sedimentation of IBV RNA in aqueous gradients. (a) RNA was extracted from IBV purified by differential and velocity centrifugation and centrifuged on a 12-ml 15 to 30% linear sucrose-SDS gradient for 3.5 h at 20°C and 200,000 × g. Samples (30 μl) were taken for the determination of radioactivity (▲). The remainder of the fractions were precipitated in the presence of 100 μg of carrier RNA with 70% ethanol at −20°C, dissolved in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), and treated with 20 μg of RNase per ml for 20 min at 25°C. The trichloroacetic acid-precipitable radioactivity was then determined (△). (b) IBV RNA was centrifuged on isokinetic sucrose-SDS gradients with a top concentration of 15% (wt/vol) for 5.5 h at 5°C and 200,000 × g. Gradients contained 100 mM (●) or 1 mM (○) LiCl.*

ior of IBV RNA under denaturing conditions was studied using sucrose-formamide gradients. Attempts to use dimethyl sulfoxide as a chaotropic agent were unsuccessful because this agent caused aggregation of IBV RNA. A similar effect of dimethyl sulfoxide on Sindbis virus RNA has been reported (24). When ^{3}H-labeled IBV RNA was centrifuged in formamide-sucrose gradients in parallel with Sindbis virus RNA and 28S and 18S rRNA markers, little, if any, dissociation of IBV RNA occurred (Fig. 4a). This suggests that IBV RNA consists of a single continuous polynucleotide chain. When IBV and Sindbis virus RNAs were recovered from the formamide-sucrose gradient and recentrifuged on aqueous gradients, all of the Sindbis virus RNA sedimented at 47S (see previous section), and most (80%) of the IBV RNA sedimented at 64S. However, about 20% of the IBV RNA sedimented at 50S to 60S as a rather broad shoulder.

When the logarithm of the molecular weight of each of the marker RNAs was plotted against the logarithm of the distance sedimented in the formamide-sucrose gradient (24), a straight line was obtained (Fig. 4b). From this calibration graph, the molecular weight of IBV RNA was estimated to be about 8.0×10^6, a value similar to that obtained by isokinetic centrifugation under aqueous conditions (see previous section).

As an alternative to gradient centrifugation, the molecular weight of IBV RNA was determined by agarose gel electrophoresis in the presence of the denaturant methylmercuric hydroxide (1). On these gels, IBV RNA electrophoresed as a single discrete peak (Fig. 5a). As observed by Bailey and Davidson (1), a linear

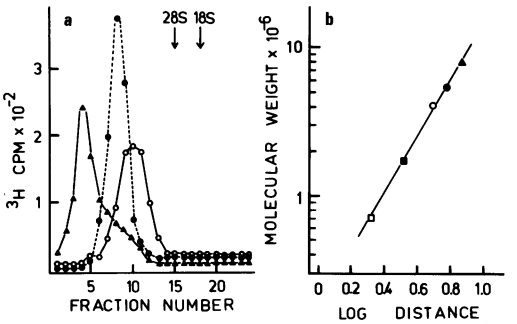

FIG. 3. *Determination of the sedimentation coefficient of IBV RNA. (a) IBV (▲), NDV (●), and Sindbis virus (○) RNAs were centrifuged in the presence of ^{14}C-labeled rRNA's on 12-ml isokinetic sucrose-SDS gradients as described in the legend to Fig. 2. Samples (50 μl) were analyzed for radioactivity. (b) Estimation of the molecular weight of IBV RNA by plotting S values obtained in an aqueous gradient against the logarithm of the distance RNAs sedimented in the centrifuge tubes. Symbols: IBV RNA (▲), NDV RNA (●), Sindbis virus RNA (○), 28S RNA (■), and 18S rRNA (□).*

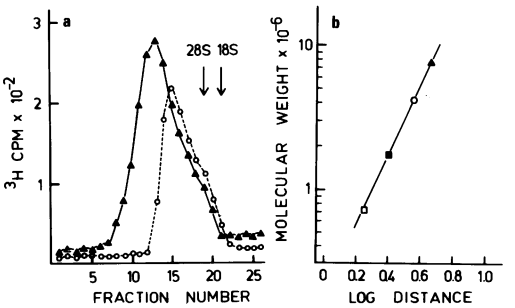

FIG. 4. *Sedimentaion of IBV RNA in formamide-sucrose gradient. (a) RNA samples were taken up in 70% formamide containing RNA buffer, kept for 30 min at 25°C, and then spun for 5 h at 200,000 × g and 12°C. Symbols: IBV RNA (▲), Sindbis virus RNA (○). (b) Estimation of the molecular weight of IBV RNA by plotting S values obtained in a formamide gradient against the logarithm of the distance RNAs migrated and sedimented in the centrifuge tubes. Symbols: IBV RNA (▲), Sindbis virus RNA (○), 28S rRNA (■), and 18S rRNA (□).*

relationship was obtained between the logarithm of the molecular weight of the marker RNAs and their electrophoretic mobility (Fig. 5b). From this relationship, the molecular weight of IBV RNA was estimated to be 8.1 ± 0.2 × 10^6.

Oligonucleotide fingerprinting of IBV RNA. Although sedimentation analysis and gel electrophoresis gave similar values for the molecular weight of IBV RNA, these values were obtained by extrapolation of calibration curves. Extrapolation was unavoidable since no suitable marker RNA of an established molecular weight greater than that of IBV RNA is presently available. To overcome this limitation, we considered it important to determine the molecular weight of IBV RNA by a technique that does not depend on properties of the intact molecule. One such technique is based on the relative (to total applied) radioactivity in characteristic oligonucleotides derived from single-stranded RNAs after complete RNase T_1 digestion (2). The application of this technique to ^{32}P-labeled IBV RNA and marker Semliki Forest virus RNA is shown in Fig. 6. In both fingerprints the pattern of oligonucleotide spots above the dashed line is relatively characteristic (13). Since the number of characteristic spots is proportional to the molecular weight of the RNA, it is clear by inspection that the molecular weight of IBV RNA considerably exceeds that of Semliki Forest virus RNA (estimated to be 4.2 ± 02. × 10^6). To determine the molecular weight of IBV RNA, several characteristic oligonucleotide spots were recovered, counted, and digested with pancreatic RNase, and their products were characterized by high-voltage electrophoresis with markers of known structure (3). When the criteria detailed by Billeter et al. (2) were used, seven pure T_1 oligonucleotides from IBV RNA were identified. Properties of these seven oligonucleotides are shown in Table 1 together with data on six pure oligonucleotides from Semliki Forest virus RNA. From the composition of these oligonucleotides (arrows in Fig. 6), their nucleotide chain length was calculated, and knowing the radioactivity in each oligonucleotide and the total recovered radioactivity in the entire gel (obtained by drying the gel and counting), estimates were made of the nucleotide complexity of each RNA (19). For IBV RNA the complexity was calculated to be 23,170 ± 920 nucleotides, and for Semliki Forest virus RNA it was calculated to be 12,570 ± 480 nucleotides (Table 1). These complexities correspond to molecular weights of 8.11 ± 0.32 for IBV RNA and 4.40 ± 0.17 × 10^6 for Semliki Forest virus RNA. This latter value is in close agreement with previous estimates of

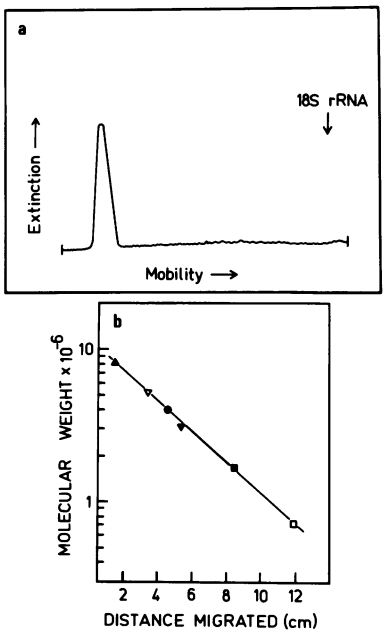

FIG. 5. *Electrophoresis of IBV RNA on denaturing agarose gels. IBV RNA* (▲) *together with marker Sendai virus RNA* (▽), *Semliki Forest virus RNA* (●), *poliovirus RNA* (▼), *28S* (■), *and 18S rRNA's* (□), *all labeled with [^{32}P]orthophosphate and prepared as described in the text, was electrophoresed on a 1% agarose slab gel containing 4 mM methylmercuric hydroxide. After electrophoresis, which was at 80 V for 16 h, the gel was dried and autoradiographed for 43 h. (a) Densitometer tracing of the lane containing IBV RNA. (b) Plot of the electrophoretic mobility of each RNA species against its molecular weight.*

the molecular weight of Semliki Froest virus RNA and, as such, substantiates the estimate of the molecular weight of IBV RNA. Two other points emerge from these fingerprints. First, both fingerprints have a streak in the top left-hand corner. Since the streak in the Semliki Forest virus RNA fingerprint is known to be the 3'-terminal poly(A) tract and since many other polyadenylated RNAs give similar streaks, it is likely that the streak in the IBV RNA fingerprints is poly(A). This would confirm the observation based on binding to oligodeoxythymidylic acid-cellulose that IBV RNA contains a poly(A) tract (Lomniczi, in press). Second, in addition to the characteristic oligonucleotides of IBV RNA whose composition is given in Table 1, the partial or almost complete composition of an additional 37 oligonucleotides has been determined (data not shown). About 32 of these are probably pure (2), bringing the total of unique characteristic oligonucleotides to about 40. In other words, the great majority of characteristic oligonucleotides of IBV RNA

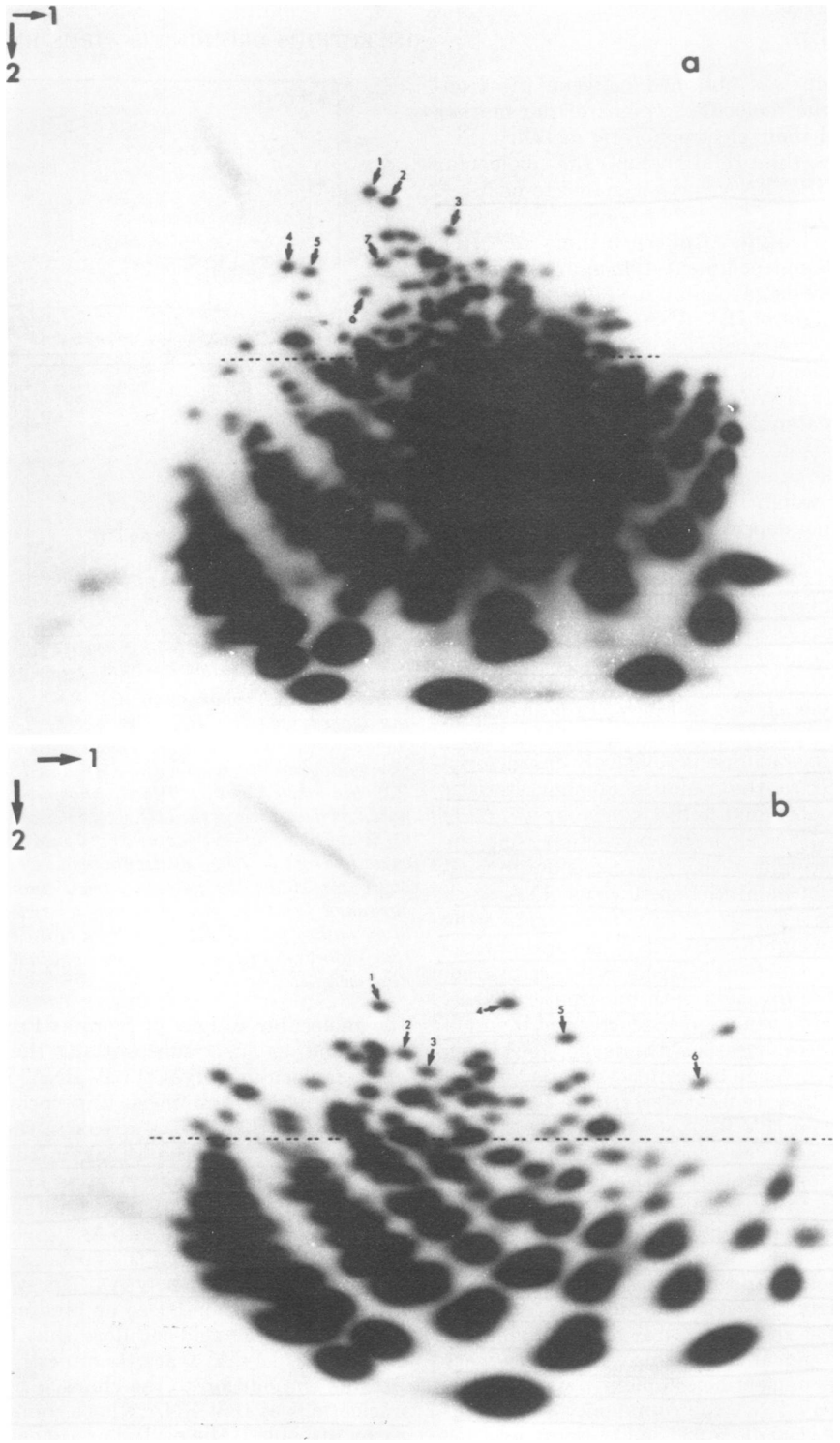

FIG. 6. *Oligonucleotide fingerprint of IBV RNA.* ^{32}*P-labeled (a) IBV RNA (25 × 10^6 cpm) and (b) Semliki Forest virus RNA (20 × 10^6 cpm), prepared as described in the text, were digested to completion with RNase* T_1*, and the resultant nucleotides were fractionated by two-dimensional polyacrylamide gel electrophoresis. The first dimension (left to right) was a 10% gel containing 6 M urea at pH 3.5, and the second dimension (top to bottom) was a 20% gel at pH 8.3. After electrophoresis, the gels were autoradiographed for 17 h, and the arrowed oligonucleotides were recovered for further characterization. The patterns of oligonucleotides above the dashed lines are considered to be characteristic (7).*

TABLE 1. *Calculation of the nucleotide sequence complexity of IBV and Semliki Forest virus RNAs from the yield of pure oligonucleotides*

RNA	Oligonucleotide no.	Composition[a]	Chain length (nucleotides)	Radioactivity (cpm)	Complexity[b] (nucleotides)
IBV	1	1.0 A_3C, 2.1 A_2U, 2.0 A_2C, 1.1 AC, 1 G, 10.0 U, 12.2 C	41	30,981	21,174
	2	1.1 A_3C, 1.0 A_2U, 3.0 A_2C, 2.1 AC, 1 G, 9.9 U, 8.0 C	39	25,692	24,287
	3	1 A_2G, 0.9 A_3C, 3.0 A_2C, 3.1 AC, 3.1 U, 2.1 C	27	20,695	20,874
	4	1.1 A_3C, 2.1 A_2C, 1 G, 2.1 U, 3.1 C	16	13,170	19,438
	5	1 AG, 3.1 AU, 3.1 U, 4.2 C	15	9,298	25,810
	6	1.0 A_3C, 2.1 A_2C, 1 G, 1.1 AC, 1.1 U	13	8,988	23,141
	7	1.0 A_3C, 2.2 A_2U, 2.0 A_2C, 1 G, 1.1 U, 1.2 C	19	11,068	27,466
Avg ± SEM[c]					23,170 ± 920
Semliki Forest virus	1	1.1 A_3C, 3.0 A_2U, 2.2 AC, 1 G, 3.1 U, 7.1 C	28	25,502	13,175
	2	1.2 A_4C, 2.9 A_2U, 1 A_2G, 0.9 U	18	14,720	14,674
	3	1 AG, 2.1 A_2U, 1.9 AC, 1.1 U, 1.9 C	16	17,279	11,112
	4	1.2 A_2C, 3.1 A_2U, 2.1 AC, 1.8 AU, 1 G, 3.1 U, 2.2 C	26	26,575	11,740
	5	1 AG, 6.3 AU, 2.0 AC, 1.1 U, 2.1 C	21	19,883	12,674
	6	2.9 A_2U, 1.2 AC, 1 G, 2.1 U	14	13,948	12,045
Avg ± SEM					12,570 ± 480

[a] Average of two determinations.

[b] Calculated from the equation: complexity = [chain length of nucleotide × total recovered counts per minute (16×10^6 for IBV RNA; 12×10^6 for Semliki Forest virus)]/radioactivity in the nucleotide.

[c] SEM, Standard error of the mean.

are present only once per molecule, suggesting that little, if any, sequence reiteration is present in the IBV genome.

DISCUSSION

The determination of the size of single-stranded RNAs larger than about 3×10^6 daltons may lead to conflicting results if examinations are based exclusively on hydrodynamic or electrophoretic properties. This can be illustrated by reference to the RNAs of the paramyxoviruses, notably NDV and Sendai virus, for which figures of 2.3×10^6 to 7.5×10^6 have been reported for the molecular weight and 48S to 57S for the sedimentation coefficient (7, 17). Recently RNA length measurements determined by electron microscopy have partially overcome many of the problems associated with the aforementioned parameters. By this technique the molecular weight of paramyxovirus RNA has been established as 5.2×10^6 to 5.8×10^6 (4, 16). However, electron microscopy, although providing useful information on the organization of viral genomes, does not afford data on genetic complexity. This aspect of viral nucleic acids can be investigated by a variety of techniques, including molecular hybridization and oligonucleotide fingerprinting.

In the present paper we have examined the size and genetic content of IBV RNA after gentle extraction with proteinase K in the presence of SDS (11) followed by phenol extraction and gradient centrifugation. We adopted this procedure, first, because it consistently gave RNA that sedimented at 64S with no evidence of fragmentation and, second, because we wished to exclude the possibility of covalent protein cross-linking either within or between RNA strands. Our attempts to use phenol and SDS without proteinase K frequently resulted in the degradation of the 64S RNA. This may explain the origin of the extremely heterogeneous RNA species previously isolated from IBV (25). When both nondenaturing isokinetic gradients (Fig. 3) and denaturing standard gradients (Fig. 4) were used, a linear relationship was observed between the logarithm of the molecular weight and the logarithm of the sedimentation distance. This relationship covered the molecular weight range from 0.6×10^6 to

5.6×10^6. Both of these approaches together with analysis on methylmercuric hydroxide-containing gels (1) gave a value of about 8×10^6 for the molecular weight of IBV RNA. Support for a value of about 8×10^6 daltons was convincingly provided by the oligonucleotide fingerprinting data, and taking this value together with the established single strandedness and poly(A) content of IBV RNA (Lomniczi, in press) leads us to the conclusion that the genome consists of a single continuous polynucleotide chain of positive polarity consisting of about 23,000 mononucleotides. This would establish IBV RNA as the largest viral RNA genome described to date.

Analysis under denaturing conditions on both gradients and gels showed no evidence of a subunit structure for IBV RNA. However, although methylmercuric hydroxide is an extremely powerful chaotropic agent, we cannot rigorously exclude the possibility that the IBV genome is segmented. In any event, the results reported here are not consistent with the observations of Garwes et al. (9) that purport to show that the genome of hemagglutinating encephalomyelitis virus and transmissible gastroenteritis virus, two other coronaviruses, can be dissociated to give 35S and 4S RNA.

If one assumes that most of the IBV genetic information is expressed in infected cells, then IBV RNA would code for about 800,000 daltons of protein or between 12 and 15 average-sized polypeptides. Preliminary experiments indicate that IBV contains up to nine structural proteins that together account for about 40% of this coding potential. Presumably the remainder codes for nonstructural proteins, including the enzyme(s) required for IBV RNA synthesis. Although not impossible, it would seem unlikely that the entire IBV genome is translated to a giant polyprotein of a molecular weight of approximately 800,000, which then undergoes cleavage. What seems more likely, particularly in view of the strategy of expression of the genome of other large plus-stranded RNA viruses (14), is the transcription of one or more subgenomic, possibly polycistronic mRNA's, each encoding proteins with related functions. Experiments with cell-free systems programmed with IBV RNA and an analysis of IBV-specified proteins and RNAs in infected cells should illuminate this question.

ACKNOWLEDGMENT

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