Ultrastructural Characterization of Genome of Epizootic Hemorrhagic Disease Virus

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Purified epizootic hemorrhagic disease (EHD) virus preparations were treated with urea and sodium perchlorate. The viral ribonucleic acid (RNA) released when spread on protein monolayers according to the Kleinschmidt technique was examined by rotary shadow-casting electron microscopy. The viral RNA released by urea treatment had filaments which frequently formed three to five loop-shaped figures of varied length. In 80% of the virus particles the lengths of the viral RNA released were 2.5 to 4.5 μm. The sodium perchlorate-released viral nucleic acid also appeared linear, and about 70% had lengths of 0.1 to 2.0 μm, the longest filament measuring 5.8 μm. Evidence was obtained that EHD virus contains double-stranded RNA as its genetic material and the molecular weight of the EHD viral RNA was calculated to be $1.22 \times 10^6$ to $1.51 \times 10^6$ daltons.

Since epizootic hemorrhagic disease (EHD) was first reported by Shope et al. (25, 26), the properties of the virus and viral pathogenesis in its natural host, the white-tailed deer, have been the subjects of several publications (7, 14, 27, 28, 29, 30, 36). The viral morphogenesis has been studied in detail in the brains of newborn mice as well as in BHK-21 cells. The development of EHD virus was closely associated with intracytoplasmic viral matricees and tubular structures (29). Similar viral morphogenesis has also been reported in cells infected with reoviruses and bluetongue virus (1, 4, 5, 11, 21). In a pathogenesis study in deer experimentally infected with EHD virus, it was demonstrated that vascular endothelial cells are target cells for EHD virus replication (30). These observations suggest that viral injury to endothelial cells may initiate a series of events, including intravascular thrombosis, leading to hemorrhages (30). By negative-contrast electron microscopy it has been shown that EHD virus has icosahedral symmetry with a size ranging from 58 to 62 nm in diameter and that it probably contains double-stranded RNA as its genome (29). Although various taxonomic characteristics have been used in the classification of animal viruses, the double-stranded nature of its nucleic acid has been suggested as a fundamental property of certain RNA viruses (21, 33). The double-stranded RNA of reoviruses has been extensively studied (2, 3, 9, 10, 16, 17, 31). In an electron microscope study, Vasquez and Kleinschmidt (31) have shown that fragments of viral genomes were released from reovirus particles upon treatment with urea. In the present study, available criteria and methods for examining the double-stranded nature were used in attempts to characterize the genome of EHD virus. The results were compared with those of other viruses similar to EHD virus.

MATERIALS AND METHODS

**Viruses**. The Alberta strain of EHD virus (36) was used throughout this work. The virus studied was either passaged in mice less than 3 days old, inoculated by the intracerebral route (sixth to eighth passages), or further propagated in baby hamster kidney cells (BHK-21) for three to five passages.

**Cell Culture**. The BHK-21 cells, clone 13, originally established by MacPherson and Stoker (18), were purchased from Grand Island Biological Co., Grand Island, N.Y. Cells were grown as stationary cultures in prescription bottles, Blake bottles, or Leighton tubes, depending on the experiment. Growth medium consisted of Eagle minimum essential medium in Hanks balanced salt solution (HMEM) supplemented with 5% fetal calf serum (FCS), streptomycin sulfate (100 μg/ml), penicillin G potassium (250 infective units/ml), and tyrocidine (60 μg/ml). For maintaining monolayer cell sheets the FCS concentration was reduced to 1%.

**Nucleic acid determination: viral inhibition studies**. Cell cultures were pretreated for 24 h with 5-bromo-2'-deoxyuridine (BUDr) at 30 μg/ml or 5-fluoro-2'-deoxyuridine (FUDR) at 0.25 μg/ml in HMEM (11). Immediately before virus inoculation, the growth medium was removed, and pretreated and untreated...
cultures were seeded with 0.1 ml of the respective dilutions of virus. After an adsorption period of 1 h at 37°C, the cultures were washed with HMEM and finally 1 ml of maintenance medium (HMEM plus 1% FCS), with or without BUdR or FUdR, was added to the respective culture tubes. Infectivity titrations were carried out after 48 to 72 h when cultures showed advanced cytopathic effects. The experiment was repeated once with the same procedures. Adenovirus type 2 and reovirus type 3 were used as known deoxyribonucleic acid (DNA) virus and ribonucleic acid (RNA) virus controls, respectively.

Virus purification and buoyant density determination. A modified method was used based on the methods of Bellamy et al. (3) and Verwoerd (32). The cells and medium were transferred to a centrifuge bottle (approximately 200 ml) and centrifuged for 1 h at 4080 x g. Each pellet was resuspended in 10 ml of homogenization buffer containing 0.01 M tris(hydroxymethyl)aminomethane, 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetate, pH 7.4, and homogenized for 90 s in a mixer. The mixture was then centrifuged for 15 min at 30,000 x g in the 30 rotor in a Spinco model L ultracentrifuge, and the supernatant fluid was collected. The resulting pellets were resuspended in 10 ml of homogenization buffer and sonicated for 30 s with a Blackstone ultrasonic unit; debris was centrifuged once again for 15 min at 30,000 x g, and the supernatant fluid was decanted.

The combined supernatant fluids were mixed with half of a volume of fluorocarbon (Freon 113, trichlorotrifluorothane) and sonicated as above for 2 min. The mixture was centrifuged to separate the phases (3,000 x g, 15 min), and the upper aqueous phase containing the virus was removed. The lower Freon phase and interphase were re-extracted with 10 ml of homogenization buffer, sonicated for 2 min, and centrifuged. The combined supernatant fluids containing the virus were centrifuged for 2 h at 100,000 x g in the 50 rotor in a Spinco model L ultracentrifuge. The pellets were resuspended in a small volume of homogenization buffer, sonicated, and layered over 4-ml volumes of preformed cesium chloride (CsCl) solutions ranging in concentration from 30 to 60%. Centrifugation was carried out at 78,000 x g for 1.5 h in the SW 39 rotor of the Spinco model L ultracentrifuge. After centrifugation, the bottom of the centrifuge tube was punctured in a Buchler fraction collector and 10-drop fractions were collected. Each fraction was examined for virus particles in an electron microscope by the negative-staining method (29). Buoyant density measurements were made on virus-containing fractions with a densitometer. The densitometer was calibrated by plotting refractive-index reading against densities of CsCl solution determined by weight-volume measurements.

Treatments of virus with urea and sodium perchlorate. The EHD virus preparations were mixed with equal volumes of 4, 6, or 8 M urea at 4°C or room temperature for 5, 10, or 15 min. To 0.1 ml of urea-treated samples, 0.1 ml of a 1 M ammonium acetate solution containing 0.01% chymotrypsin (Nutritional Biochemical Corp.) was added. The mixture was immediately allowed to flow down an inclined glass surface onto a hypophase of 0.3 M ammonium acetate, pH 7.0 (8).

Some EHD virus preparations were treated with 1.5 M sodium perchlorate (pH 7.8) at room temperature for 5 to 20 min. Then, an equal volume of 1 M ammonium acetate containing 0.01% chymotrypsin was added and immediately the mixture was spread on a hypophase as described above.

Both urea- and sodium perchlorate-treated samples were then subjected to rotary shadow-casting electron microscopy.

Extraction of viral RNA from purified virus preparations. The method described by Scherrer and Darnell (23) was followed for the isolation of viral RNA. Purified virus from a CsCl gradient was dialyzed against homogenization buffer and diluted 10-fold in 0.1 M sodium acetate buffer (pH 5.0). Sodium dodecyl sulfate was added to 1% final concentration and the contents were mixed. An equal volume of liquefied phenol (90%), preheated to 60°C, was added, and the solution was vigorously shaken until the viscosity of the solution dropped substantially. The extraction mixture was shaken vigorously at 60°C for another 5 min. The solution was then quenched in a cryostat at about -20°C until the viscosity of the solution rose and some phenol crystals appeared. The mixture was transferred into polypropylene tubes, and the phases were separated by centrifugation at 4,080 x g for 5 min. The phenol layer was removed from below by penetrating the aqueous phase with an 18-gauge, 4-in needle mounted on a 50-ml syringe.

After resuspension of the interphase, the aqueous layer was transferred back to the extraction flask. More preheated liquefied phenol was added, and the mixture was shaken at 60°C for another 5 min. After centrifugation, removal of phenol, and resuspension of the interphase, the aqueous phase was extracted once more as described above. Residual phenol in the aqueous phase was removed by two extractions with ether.

The solution was allowed to precipitate for a period of 16 to 18 h at -20°C after adding 2 vol of -20°C ethyl alcohol (95%). The precipitate was separated by centrifugation for 30 min at 4,080 x g. The ethyl alcohol was drained and the remaining ethyl alcohol was evaporated by short exposure to high vacuum. The sample was dissolved in SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate. The sample prepared in this manner was used for the thermal denaturation test, the RNase sensitivity test, and rotary shadow-casting electron microscopy examination.

Thermal denaturation test. In order to further characterize EHD virus RNA, the heat stability of extracted viral RNA was determined. Samples of extracted viral RNA dissolved in 0.1 x SSC, pH 7.2, were heated in quartz cuvettes in a thermostatically controlled Beckman spectrophotometer, and the absorbancy at 260 nm was determined at various temperatures according to the methods of Marmur and Doty (19).

RNase sensitivity test. Samples of extracted viral RNA in 0.1 x SSC were treated with ribonuclease-A type 1-A (10 µg/ml; Sigma Chemical Co., tested for
The scope which the viral nucleic acid molecules examined for was sample nucleic acid ethanol wire of ported and Co., below.

The results obtained indicate that the treatments with BUdR and FUdR were not demonstrated that the treatments with BUdR and FUdR did not inhibit the replication of reovirus, whereas the replication of adenovirus was inhibited by treatment with

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<th>Treatment</th>
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<td>EHD virus</td>
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<td>BUdR (30 μg/ml)</td>
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* Titer = mean tissue culture infective dose per 0.1 ml.

activity on commercially obtained RNA, Sigma Chemical Co., type XI purified) for 15 min at 27 C, and the RNase was removed by phenol extraction. A second sample was similarly treated but the RNase was omitted. The treated and untreated samples were examined for absorbency at 260 nm in a spectrophotometer and for ultrastructure in an electron microscope by the rotary shadow-casting method described below.

Rotary shadow-casting electron microscopy.

The method of Kleinschmidt (15) was followed. The spreading chymotrypsin, as it formed a monolayer in which the viral nucleic acid molecules were trapped, was picked up on carbon-coated Formvar films supported on 200- or 300-mesh copper grids. The grids were then rinsed with distilled water for 30 s. The adherent water droplets were replaced by absolute ethanol and allowed to dry.

To enhance contrast and allow the thread-like nucleic acid molecules to be visualized clearly over their length, the grid was shadowed on a rotary turntable at an angle of 10° with 8 mg of platinum-palladium alloy wire evaporated from a 0.8-mm tungsten wire at a mean distance of 8.5 cm from the grids in an evaporating unit.

The grids examined in a Philips EM 200 electron microscope at an original magnification of 9,200 were recorded on 35-mm film or at 23,000 magnification, on 3.5 × 4 photographic plate. The magnification was calibrated with a Ladd carbon grating replica (55,864 lines/in). The lengths of the molecules were measured, either on positive prints or on tracings obtained by projecting the photographic plates onto a piece of paper, with a map measure. Molecular weight estimations were calculated by the methods of Arnott et al. (2), of Bellamy et al. (3), and of Langridge and Gomatos (17).

RESULTS

Nucleic acid determination: virus inhibition studies. The results presented in Table 1 demonstrated that the replication of EHD virus was not inhibited by treatment with BUdR (30 μg/ml) and FUdR (0.25 μg/ml). A known RNA virus, reovirus type 3, and a known DNA virus, adenovirus type 2, were used as controls. The results obtained indicate that the treatments with BUdR and FUdR did not inhibit the replication of reovirus, whereas the replication of adenovirus was inhibited by treatment with FUdR. Some RNA viruses which depend on DNA synthesis for the production of new virus progeny might also be inhibited by BUdR or FUdR. However, no nonenveloped, cubically symmetrical RNA virus is known to be inhibited by BUdR or FUdR. The results of these experiments, therefore, would imply that the EHD virus genome is composed of RNA.

Virus purification: buoyant density determination. The data obtained from a representative experiment are summarized in Fig. 1. The results indicate that the greatest concentration of virus was collected in fraction 11 as measured by absorption at 260 nm and estimation of virions by electron microscopy. The peak of concentration, as measured with a densitometer, was observed at 1.38 g/ml. Electron microscope examinations of fractions 10 to 12 generally showed virus particles free of cellular elements while fractions 16 and 18 showed mem-
FIG. 2. Electron micrograph illustrating a high magnification of EHD virus particles. The surfaces of full capsids were composed of capsomeres which contain an axial hole of about 4 nm in diameter. The width of each capsomere measured about 11 nm. Each capsomere consists of 5 to 6 subunit structure (arrows). Negative contrast staining. Scale line = 50 nm.
Fig. 3. Rotary shadow-casting electron micrographs of EHD virus particles which were treated with 6 M urea at room temperature for 15 min. The virus particles are seen in the process of releasing their nucleic acid molecules. The total length of the released molecules measured about 3.2 μm for a and 3.8 μm for b. Scale line = 0.5 μm.
brane-like material associated with virus particles.

**Virus structure.** Measurements, performed on 300 purified virus particles, indicated that the average diameter of EHD virus was 60.3 nm. Approximately 90% of the particles examined had diameters between 58 and 63 nm, whereas only 6% had diameters between 52 and 57 nm. These results reaffirm our previous electron microscope findings that EHD particles had diameters ranging between 58 and 62 nm (29). The surfaces of full capsids were composed of capsomeres which contained an axial hole of about 4 nm in diameter. The width of the capsomeres measured about 11 nm. In a few instances each capsomere was observed to consist of 5 to 6 subunit structures (Fig. 2).

**Treatment of virus preparation with urea.** At first attempt, the virus preparation was treated with 4 M urea at 4 C for 5 min, but there was no indication of release of viral genome. The rotary-shadowed virus particles appeared as a hexagonal profile probably representing icosahedral capsids. The virus preparation was then treated with 6 and 8 M urea at room temperature for 10 to 15 min. When these preparations were examined in the electron microscope, most viral particles were found in the process of releasing filaments which were assumed to be viral RNA molecules. The evidence for this assumption will come later. Figures 3a and b are representative micrographs in which the released filaments are seen to be attached to the particles, forming spider-like structures. The released filaments frequently formed 3 to 5 loop-shaped figures of varied

![Graph showing RNA filament release](image)

**Fig. 4.** RNA filament release, measured in total length per “spider” after spreading of EHD virus particles on 6 M urea. The histogram represents the combined length measurements of all the RNA filaments of each “spider”, given in percentage of “spider”.

![Micrographs of EHD virus particles treated with 1.5 M sodium perchlorate](image)

**Fig. 5.** Rotary shadow-casting electron micrographs of EHD virus particles treated with 1.5 M sodium perchlorate for 5 min at room temperature. The released RNA molecules are linear and frequently one filament per particle. Scale line = 100 nm.
length (Fig. 3). Figure 4 shows the total length per "spider" after spreading of EHD virus particles on 6 M urea for 15 min. Of 92 "spiders" measured, 25% had total lengths between 3.0 and 3.5 μm, 23% had total lengths between 3.5 and 4.0 μm, and 18% had total lengths between 4.0 and 4.5 μm (Fig. 4).

**Treatment of virus preparation with sodium perchlorate.** When the EHD virus preparation was treated with 1.5 M sodium perchlorate for about 5 min at room temperature and mounted monolayers were rotary shadowed, the released RNA molecules were short and frequently one or two filaments per particle were observed (Fig. 5a–f).

When an EHD virus preparation was treated with 1.5 M sodium perchlorate at room temperature for about 20 min and then spread on the hypophase, filaments of various lengths were seen released from the particles, frequently one or two filaments per particle. The released viral nucleic acid appeared linear and uniform in width. The majority (more than 80%) of the released filaments had total lengths between 0.1 to 2.0 μm. However, filaments longer than 2.0 μm were also observed (Figs. 6a, b). The longest viral RNA filament observed was 5.8 μm (Fig. 6b). Figure 7 shows the distribution of lengths of RNA filaments released under the condition described above. Three peaks, at 0.3 to 0.4, 0.6 to 0.7, and 1.2 to 1.3 μm, are presented in the histogram (Fig. 7).

**Extraction of viral nucleic acid from purified virus preparation.** The extracted viral nucleic acid, examined by rotary shadow-casting electron microscopy, appeared as linear filaments of uniform width and various lengths (Fig. 8a–j). The frequency distribution of 204 molecules of viral nucleic acid is shown in Fig. 9. The molecules varied in length from about 0.1 to 1.8 μm. Filaments longer than 1.8 μm were not observed in this series of observations. The majority (90%) of the filaments were found to have lengths varying from 0.1 to 1.0 μm. Two small peaks, at 0.2 to 0.3 μm and 0.4 to 0.5 μm, are depicted in the histogram (Fig. 9).

**Thermal denaturation test.** The change in absorbency of EHD virus RNA occurred when the temperature was increased from 70 to 102 C, with a sharp increase at 87 C, and the Tm was approximately 94 C (Fig. 10). This result corresponds well with the thermal denaturation of

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Fig. 6. Rotary shadow-casting electron micrographs of EHD virus particles treated with 1.5 M sodium perchlorate for about 20 min at room temperature. The released RNA molecules are linear and measured 5.0 μm for a and 5.8 μm for b. Scale line = 1 μm.
Fig. 7. RNA filament release, measured in total length per particle after spreading EHD virus particles on 1.5 M sodium perchlorate for 20 min at room temperature. The histogram represents the total length measurements of RNA filaments of each particle, given in percentage of particles.

double-stranded RNA from reovirus (10).

**Effect of RNase on EHD virus RNA.** Samples of extracted viral RNA in 0.1 x SSC, when exposed for 15 min to pancreatic RNase (10 μg/ml), showed no hyperchromic effect at 260 nm or at any other wavelength in the ultraviolet region. To test the activity of RNase used, a commercially obtained ribonucleic acid (Sigma Chemical Co. type XI, purified), treated with RNase at the same concentration for 15 min at 27°C, showed a marked hyperchromic effect. This result indicates that the RNase used was effective for single-stranded RNA. In one experiment, the samples with and without RNase treatments were examined with rotary shadow-casting electron microscopy. The results indicate that the viral RNA components were relatively resistant to degradation by RNase. Figures 1a and b are histograms obtained from the treatments with and without RNase; the two histograms are similar except for the presence of a maximum of fragments of 0.2 μm after RNase treatment.

**DISCUSSION**

The release of viral nucleic acid from individual EHD virus particles after treatments with urea and sodium perchlorate has been visualized by rotary shadow-casting electron microscopy. The filaments liberated from the virus particles have been interpreted as double-stranded RNA for the following reasons. (i) Results of viral inhibition studies by BUdR and FuDr indicated that EHD virus contains RNA as its genetic material. (ii) The extracted viral RNA components were relatively resistant to degradation by RNase. (iii) The thermal denaturation curve indicates that EHD viral RNA had a high melting temperature of 94. (iv) In electron micrographs of rotary-shadowed preparations, the extracted viral nucleic acid of EHD virus appeared as linear filaments of uniform width resembling molecules of double-stranded RNA of reovirus. (v) By urea treatment similar forms of viral RNA release have been described as “spiders” in reovirus (31).

Although multiple releases of filaments from individual EHD virus particles were observed (frequently 3 to 5 releases per particle), the fragmented filaments with free distal endings as described in reovirus (31) were rarely observed in any urea-treated preparations in the present study. Therefore, it was not possible to calculate the number of fragments per “spider”. However, the total filament lengths could be measured. The longest total filament length per “spider” obtained from urea-treated samples was 4.7 μm; therefore, EHD virus may contain as much as 4.7 μm of double-stranded RNA per particle.

It is of interest that the mode of viral RNA release of EHD virus appeared different between urea and sodium perchlorate treatments. The former often gave a spider-like multiple release of circular or loop-like filaments, whereas the latter usually gave one or occasionally two filaments per particle with varied lengths of one or two free endings. Granboulan and Niveleau (12) were able to obtain a long RNA filament of 5 μm in length, and lengths up to 7.7 μm have been observed by Dunnebacke and Kleinschmidt (6) on treatment of reovirus with urea. In the present study, the urea treatment did not give a long RNA filament even though treatments were performed for longer periods of time at room temperature with 8 M urea. On the other hand, long RNA filaments were obtained by the treatment with sodium perchlorate. Therefore, perchlorate treatment is considered to be more efficient in releasing the RNA from the virion than is the urea in the present experiments.

Under various sodium perchlorate treatments, the majority of the filaments observed measured less than 2.0 μm. Within the range of 0 to 2.0, 0.4, 0.7, and 1.2 μm, RNA filaments appeared consistently and in largest number.
FIG. 8. Rotary shadow-casting electron micrographs of viral RNA extracted from purified EHD virus preparations. The molecules of viral RNA appeared to be filamentous with lengths from 0.1 to 1.8 μm. Scale line = 100 nm.
stranded RNA obtained from purified bluetongue virus was fractionated into 10 components by electrophoresis on polyacrylamide gels. The size of these components varied from $0.5 \times 10^6$ to $2.8 \times 10^6$ daltons, with a total molecular weight estimated at about $1.5 \times 10^7$ for the viral nucleic acid of bluetongue virus (34). The viral genome of bluetongue virus, however, has not been shown by electron microscopy.

The length measurement of nucleic acid molecules under rotary shadow-casting electron microscopy has been shown to be most useful for molecular weight estimations of double-stranded RNA. The molecular weight of the double-stranded RNA of reovirus has been estimated to be between $17 \times 10^6$ and $22 \times 10^6$ by the method described by Kleinschmidt (15). In the present studies, the longest RNA filament of EHD virus seen in electron microscopy measured 5.8 \mu m. The calculated molecular weights for double-stranded RNA per micron are $2.1 \times 10^6$ (3), $2.3 \times 10^6$ (17), and $2.6 \times 10^6$ (2). If these values are applicable for EHD virus RNA, 5.8-\mu m RNA of

These three filament lengths are, therefore, interpreted as stable subunits of viral RNA of EHD virus.

The viral genomes of reoviruses have been extensively studied (2, 3, 6, 9, 10, 17, 31). Three subunits of double-stranded RNA (0.35, 0.60, and 1.1 \mu m) have been determined by several investigators (6, 31). The genome of bluetongue virus has been identified as double-stranded RNA by Verwoerd et al. (34). The double-stranded RNA obtained from purified bluetongue virus was fractionated into 10 components by electrophoresis on polyacrylamide gels. The size of these components varied from $0.5 \times 10^6$ to $2.8 \times 10^6$ daltons, with a total molecular weight estimated at about $1.5 \times 10^7$ for the viral nucleic acid of bluetongue virus (34). The viral genome of bluetongue virus, however, has not been shown by electron microscopy.

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EHD virus falls between $12.2 \times 10^6$ and $15.1 \times 10^6$ daltons.

When samples of extracted viral nucleic acid were examined with rotary shadow-casting electron microscopy, linear filaments of uniform width and various lengths were observed. These viral nucleic acid filaments resemble molecules of double-stranded RNA of reovirus (6, 16). It has been suggested that the RNA filaments may have been broken by phenol extraction and manipulation, but the distribution pattern for reovirus would imply that the molecules have preferential breaking points (35). Although a number of biological findings support the hypothesis that multiple RNA fragments isolated from purified reovirus represent functionally important genome subunits, it is not known how they are correctly segregated during infection and arranged within infectious particles. It has been suggested, however, that subunits are weakly linked within the virion and remain together as a replicating complex during the virus multiplication cycle (24). In the present study, the majority (90%) of the filaments were observed to have lengths varying from 0.1 to 1.0 μm. Two small peaks (at 0.2 to 0.3 and 0.4 to 0.5 μm) are depicted in the histogram and probably represent subunits of the EHD viral genome.

Several RNA viruses including reoviruses (3, 10, 17), bluetongue virus (34), Colorado tick fever virus (13), wound tumor virus (16, 37), rice dwarf virus (20), and cytoplasmic polyhedrosis virus (22) have been demonstrated to contain double-stranded RNA as their genetic material. The taxonomic characteristics of these double-stranded RNA viruses are summarized in Table 2. The present observations provide evidence that EHD virus contains double-stranded RNA as its genome and suggest that EHD virus should be placed within the diplomavirus group which was proposed to include all double-stranded RNA viruses (33).

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LITERATURE CITED


