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Characterization of Virus-Specific RNA Synthesized in Bovine Cells Infected with Bovine Viral Diarrhea Virus

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Infection of bovine kidney cells with bovine viral diarrhea virus resulted in the synthesis of a single species of virus-specific RNA. Electrophoresis of this RNA on agarose-urea and agarose-formaldehyde gels indicated that it had a molecular weight of 2.9×10^6, corresponding to 8,200 bases (8.2 kilobases). This 8.2-kilobase RNA was resistant to RNase A treatment at 1 μg/ml but was digested at higher concentrations of RNase (10 μg/ml). Sedimentation on neutral sucrose gradients indicated that the majority of this RNA (98%) sedimented at 21S, with a small amount sedimenting at 33S. Sedimentation on formaldehyde-containing sucrose gradients resulted in the conversion of all of the RNA to the faster-sedimenting form. At no time after infection were we able to detect virus-specific RNA species of lower molecular weight than the 8.2-kilobase RNA. The implications of these findings with respect to the means of replication of various togaviruses are discussed.

Bovine viral diarrhea (BVD) virus has been identified as the causative agent of virus-induced diarrhea-mucosa disease in cattle (6, 9, 14). Infectivity with BVD virus was found to be sensitive to ether and chloroform treatment, and the genetic material of the virus consists of RNA (4, 5). The virus has been classified as a member of the Pestivirus genus of the non-arthropod-borne togaviruses (3, 6). However, little information is available concerning the molecular features of the genome and the structural components of BVD virus.

Previous studies of the RNA of BVD virus isolated from virion particles indicated that the major RNA component was a 3.2×10^6-dalton species which sedimented at 38S. Smaller components sedimenting at 31 and 24S were also detected (10). We have been studying the interaction of BVD virus with several types of bovine cells in cell culture. In this report we present the results of our studies on the intracellular BVD virus-specific RNA found in infected bovine kidney cells. Figure 1A shows a fluorogram of an agarose-urea gel containing RNA samples extracted from [^{3}H]uridine-labeled uninfected and BVD-infected MDBK cells. A single high-molecular-weight band (arrow) which was DNase resistant and specific for infected cells was seen. The synthesis of this RNA was resistant to actinomycin D treatment, although the drug inhibited the synthesis of all other RNA species. These results were obtained when cells were labeled either of 24 h postinfection, as above, when the cytopathic effect was moderate, or at 36 h postinfection when the cytopathic effect was extensive and cell lysis had begun. The same results were also obtained when BVD virus was grown in either bovine lung or bovine embryonic testicle cells (data not shown). Furthermore, when virus that was passed only once after plaquing was used to infect MDBK cells, the only virus-specific RNA species that could be identified was that shown in Fig. 1A (data not shown).

Figure 1B shows an analysis of RNA from infected and uninfected cells on an agarose-formaldehyde gel. Since these gels are more denaturing than those containing agarose and urea, they allow for a more accurate determination of RNA molecular weight. The fluorogram (Fig. 1B) indicates that only one RNA species could be detected which was both specific for infected cells and resistant to actinomycin D treatment. It migrated with an apparent molecular weight of 2.9×10^6, corresponding to 8,200 bases. It will be referred to as 8.2-kilobase (kb) RNA and presumably represents the genome of BVD virus.

Sedimentation analysis of the 8.2-kb RNA species is shown in Fig. 2. The BVD-specific RNA labeled in the presence of actinomycin D sedimented at 21S (Fig. 2A). To detect any more

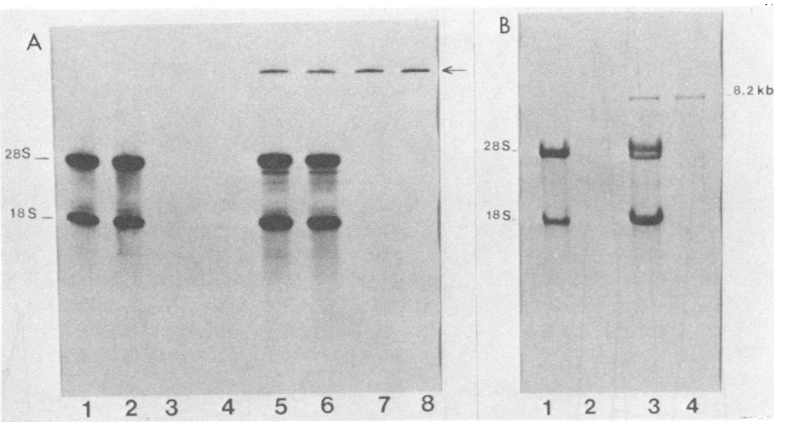

FIG. 1. Fractionation of RNA from infected and uninfected MDBK cells by gel electrophoresis. The cells used in this study were the Madin-Darby bovine kidney (MDBK) cell line, obtained from the National Veterinary Services Laboratory, University of Iowa, Ames. Cells were grown in Dulbecco modified Eagle medium containing 5% horse serum. The National Animal Disease Laboratory strain of BVD virus (obtained from D. Reed, College of Veterinary Medicine, University of Iowa, Ames) was plaque purified three times on MDBK cells and passaged twice through MDBK cells at multiplicities of 0.01 and 0.10. This twice-passed virus was then used as the stock for subsequent infections. The titer of this stock rarely exceeded 3×10^6 PFU/ml. MDBK cells were grown to 80% confluency on 100-mm tissue culture dishes and infected with 1 ml (ca. 10^6 PFU) of BVD virus. At approximately 20 to 24 h postinfection, when >80% of the cells showed moderate cytopathic effects consisting mostly of vacuolation, the culture fluid was removed and fresh growth medium either with 0.5 μg of actinomycin D per ml or without actinomycin D was added. After incubation for 60 min at 37°C, the culture fluid was then replaced with medium containing 100 μCi of [^{3}H]uridine (Amersham Corp., Arlington Heights, Ill.) per ml with or without actinomycin D. After 5 h of labeling, RNA was extracted as described previously (11). RNA samples to be treated with DNase were dissolved in 0.01 M Tris-hydrochloride (pH 7.2)–10 mM $MgCl_2$ and incubated with 20 μg of DNase I (Worthington Diagnostics, Freehold, N. J.; prepared by iodoacetate treatment [17]) per ml. for 20 min at 37°C. The samples were analyzed by electrophoresis in 1% agarose–8 M urea gels prepared in 40 mM Tris-acetate (pH 7.9)–5 mM sodium acetate–1 mM EDTA. Fluorography was performed as previously described (2), and gels were dried and exposed to Cronex 4 X-ray film at −70°C. (A) Track 1, Uninfected-cell RNA; track 2, uninfected-cell RNA treated with DNase; track 3, RNA from actinomycin D-treated uninfected cells; track 4, same as track 3 but treated with DNase; track 5, infected-cell RNA; track 6, infected-cell RNA treated with DNase; track 7, RNA from infected cells treated with actinomycin D; track 8, same as track 7 but treated with DNase. (B) [^{3}H]uridine-labeled RNA was extracted from uninfected and infected MDBK cells, treated with DNase, and fractionated on an agarose-formaldehyde gel (8). Samples treated with DNase were first extracted with phenol-chloroform-isoamyl alcohol (24:24:1, vol/vol/vol) and precipitated with 3 volumes of ethanol before being loaded onto the formaldehyde-containing gels. Track 1, Uninfected-cell RNA; track 2, RNA from uninfected cells treated with actinomycin D; track 3, RNA from infected cells; track 4, RNA from infected cells treated with actinomycin D.

rapidly sedimenting RNA, RNA was extracted from cells without actinomycin D treatment, and sedimentation was performed for a shorter time. Fractions from the regions indicated in Fig. 2B were pooled and electrophoresed on an agarose-formaldehyde gel. Fig. 2C shows that the vast majority of the 8.2-kb RNA sedimented between 18 and 28S, although a small amount sedimented ahead of 28S at about 33S. The amount of 8.2-kb RNA sedimenting in this region (which was not detectable by the technique used for Fig. 2A) varied from experiment to experiment, never exceeding 2% of the amount detected in the 21S region. We presume that the small amount of 8.2-kb RNA sedimenting at 33S on neutral sucrose gradients was due to some conformational change in the molecule which occurred during the heat treatment immediately before sedimentation.

Figure 2D shows the same type of analysis of this RNA, with sedimentation done in formaldehyde-containing sucrose gradients. All of the 8.2-kb RNA sedimented as one component just ahead of 28S. When individual fractions from this gradient were analyzed, the sedimentation value for the 8.2-kb RNA was calculated to be 33S (data not shown).

The sedimentation characteristics of the 8.2-kb RNA on neutral sucrose gradients could be due to a high degree of secondary structure which causes the RNA to sediment at 21S. Alternatively, a portion of the material could represent duplex RNA, since the 21S region is where replicative form (RF) RNA would be expected to sediment (16). True double-stranded RNA molecules are resistant to high concentrations of RNase A (10 to 20 μg/ml), and, in fact, digestion with these concentrations of RNase

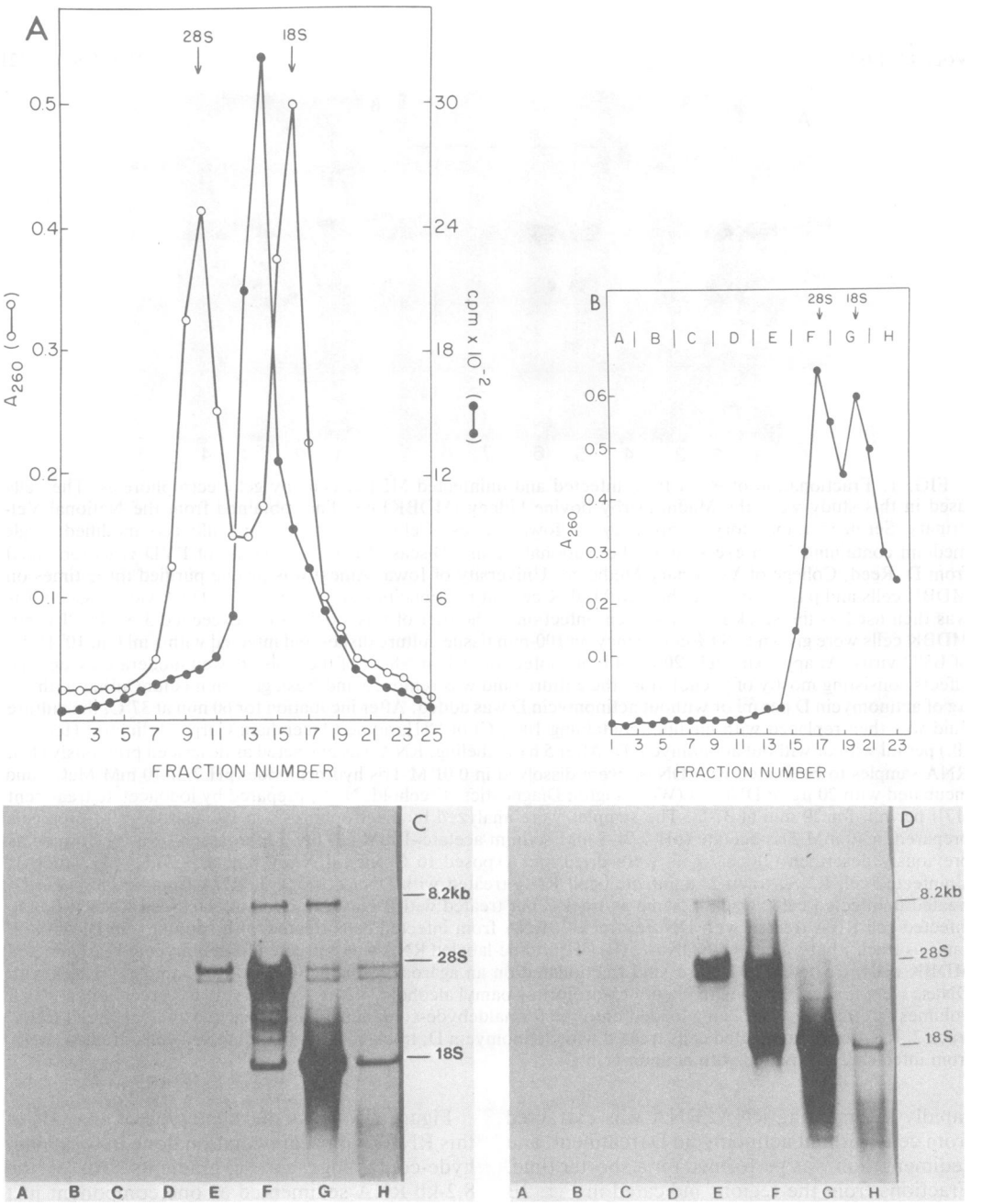

FIG. 2. Sedimentation of RNA from BVD virus-infected MDBK cells. All RNA samples to be sedimented were digested with DNase and reextracted with phenol-chloroform-isoamyl alcohol as described in the legend to Fig. 1. (A) [^{3}H]uridine-labeled RNA was extracted from infected MDBK cells treated with actinomycin D. RNA was dissolved in 0.01 M Tris-hydrochloride (pH 7.2–0.001 M EDTA–0.2% SDS, heated to 80°C for 2 min, and sedimented on a linear 5 to 20% sucrose density gradient containing 0.01 M Tris-hydrochloride (pH 7.4) and 0.1 M NaCl for 120 min at 45,000 rpm in a Beckman SW50.1 rotor at 5°C. Fractions were collected by bottom puncture (0.2 ml per fraction), and radioactivity in each fraction was determined by trichloroacetic acid precipitation (●). rRNA isolated from rabbit reticulocytes was fractionated in a parallel gradient, and the absorbance at 260 nm (A_{260}) of each fraction was determined (○). (B) Reticulocyte rRNA was sedimented in a 5 to 20% neutral sucrose gradient for 90 min as described above. Fractions were collected by bottom puncture, and the absorbance of each fraction at 260 nm was determined. (C) [^{3}H]uridine-labeled RNA from BVD virus-infected MDBK cells not treated with actinomycin D was sedimented as described above on a 5 to 20% neutral sucrose gradient for 60 min. Fractions corresponding to the regions indicated in (B) were pooled, ethanol precipitated, and fractionated on an agarose-formaldehyde gel. The gel was treated with sodium salicylate, dried, and exposed for fluorography. (D) [^{3}H]uridine-labeled RNA from BVD virus-infected MDBK cells was fractionated on a formaldehyde-containing sucrose gradient as described previously (1). Fractions corresponding to regions indicated in (A) were pooled, ethanol precipitated, and fractionated on an agarose-formaldehyde gel. The gel was fluorographed and exposed to X-ray film.

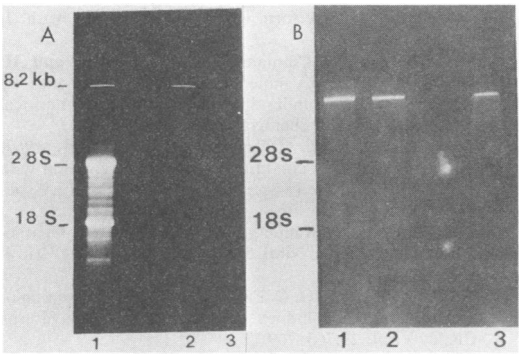

FIG. 3. RNase A digestion of BVD 8.2-kb RNA. (A) RNA extracted from BVD virus-infected MDBK cells (5 μg) was incubated with 1 μg (track 2) or 10 μg (track 3) of RNase A per ml in 0.01 M Tris-hydrochloride (pH 7.2–0.001 M EDTA–0.3 M NaCl. The reaction products were fractionated on an agarose-urea gel, stained with ethidium bromide, and photographed. Track 1, RNA incubated without RNase. (B) Poliovirus RF RNA was purified as previously described (12). Approximately 0.6 μg of RNA was incubated as described in the legend to (A) with 1.0 μg (track 2) or 10 μg (track 3) of RNase per ml, and the reaction products were fractionated on an agarose-urea gel. The gel was stained with ethidium bromide and photographed. Track 1, Undigested RNA.

has been used in their purification (7, 15). To determine whether the 8.2-kb RNA was RF-like, total RNA was prepared from BVD-infected MDBK cells and digested with various concentrations of RNase A. The products were then fractionated on agarose-urea gels, and the RNA bands were detected by staining with ethidium bromide. The 8.2-kb RNA was resistant to digestion with 1 μg of RNase A per ml, whereas all other RNA species were degraded (Fig. 3A). However, the 8.2-kb RNA was digested with 10 μg of RNase A per ml. For comparison, the RNase digestion profile of authentic poliovirus RF RNA is shown in Fig. 3B. This material was resistant to RNase A treatment at 10 μg/ml.

The 8.2-kb RNA did not bind to oligodeoxythymidylate cellulose, suggesting that it does not contain a polyadenylate tail (data not shown). However, this does not rule out the possibility that small stretches of adenosine residues exist in the molecule that are undetectable by this technique.

Infection of MDBK cells with plaque-purified BVD virus resulted in the appearance of a single species of virus-specific RNA. This RNA had a molecular weight of 2.9×10^6, corresponding to approximately 8,200 bases (8.2 kb), and was actively synthesized in the presence of actinomycin D. These values are in general agreement with those previously reported for the BVD viral genome (6). The sedimentation characteristics of the BVD virus 8.2-kb RNA were anomalous. In neutral sucrose gradients, the vast majority of the RNA (98%) sedimented at 21S (Fig. 2A and C), whereas sedimentation on formaldehyde-containing sucrose gradients revealed a single species of 8.2-kb BVD RNA sedimenting at 33S (Fig. 2D). This suggests that the BVD genome possesses a high degree of secondary structure which was apparent when the RNA was analyzed on neutral sucrose gradients but which was disrupted in the presence of formaldehyde. These sedimentation characteristics closely resemble those of an RF molecule. However, true RF RNA is resistant to RNase digestion, as shown for poliovirus RF RNA (Fig. 3B), and although the 8.2-kb BVD RNA was resistant to digestion by RNase at 1 μg/ml, it was hydrolyzed at a higher RNase concentration (10 μg/ml [Fig. 3A]). These results suggest that the 8.2-kb RNA is not a true duplex molecule. Further support for this claim was obtained from base composition analysis of the 8.2-kb RNA (23.1% U; 23.7% G, 30.8% A, 22.4% C): the high A/U ratio is not what one would expect for duplex RNA.

The results described in this report for BVD virus-specific RNA closely parallel those of Wengler et al. (16) for two flaviviruses, Uganda S and West Nile viruses. These authors found that the predominant virus-specific RNA species synthesized in infected cells was a single-stranded 42S RNA molecule. The 42S RNA of both viruses did not bind to oligodeoxythymidylate cellulose, and no subgenomic RNA species large enough to code for the precursor to the viral structural proteins was found. The authors concluded that in flavivirus-infected cells, the 42S RNA molecule codes for both the structural and the nonstructural proteins of the virus (15).

These findings raise some interesting questions concerning the replication of these togaviruses. In the best-studied cases of togavirus replication, i.e., Sindbis and Semliki Forest viruses, the genomic RNA serves as the message for the viral replicase, whereas the virion structural proteins are encoded by a subgenomic message sedimenting at 26S that consists of the 3′ one-third portion of 42S RNA (for a review, see reference 13). For BVD virus, as well as Uganda S and West Nile viruses, one RNA species apparently serves as the message for all viral proteins, both structural and nonstructural. This genomic message may program the synthesis of a large polypeptide which may then be posttranslationally cleaved into the individual virus-specific proteins. Alternatively, translation may begin at the 5′ end of the RNA and proceed to the end of the replicase gene. The replicase polypeptide may then be cleaved, al-

lowing the ribosome to proceed into the gene for the viral structural proteins. Studies on the intracellular synthesis of virus-specific proteins coupled with cell-free translation of the 8.2-kb viral RNA should help to elucidate the mechanism of BVD virus replication. These experiments are in progress.

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