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## Characterization of Berne Virus Genomic and Messenger RNAs

By ERIC J. SNIJDER,<sup>1</sup>\* JOKE EDERVEEN,<sup>1</sup> WILLY J. M. SPAAN,<sup>1</sup>  
MARIANNE WEISS<sup>2</sup> AND MARIAN C. HORZINEK<sup>1</sup>

<sup>1</sup>*Institute of Virology, Veterinary Faculty, State University of Utrecht, Yalelaan 1, 3584 CL Utrecht, The Netherlands and* <sup>2</sup>*Institute of Veterinary Virology, Veterinary Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*

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### SUMMARY

From 380S particles of Berne virus (proposed family *Toroviridae*) one species of polyadenylated RNA was isolated. Using agarose gel electrophoresis its length was estimated as 20 kb or greater. When assayed under hypertonic transfection conditions genomic RNA was found to be infectious; RNase treatment destroyed the infectivity. The positive polarity of the molecule was confirmed by filter spot hybridization using cDNA prepared against poly(A)-selected RNA from infected cells. In embryonic mule skin cells infected with Berne virus the presence of five virus-specific, polyadenylated RNA species of 7.5, 2.1, 1.4, 0.8 and at least 20 kb was demonstrated. *In vitro* translation of the 7.5, 2.1 and 0.8 kb RNAs followed by immunoprecipitation showed that they encode a 151K product (possibly the precursor to the peplomer proteins), the envelope protein and the nucleocapsid protein, respectively.

### INTRODUCTION

Berne virus (BEV) of horses is a representative of a recently defined group of vertebrate RNA viruses, for which the family status and the name *Toroviridae* have been proposed (Horzinek & Weiss, 1984; Horzinek *et al.*, 1987); antigenically related toroviruses have been demonstrated in cattle (Woode *et al.*, 1985) and humans (Beards *et al.*, 1984). The present state of knowledge regarding the toroviruses has been reviewed recently (Weiss & Horzinek, 1987; Horzinek *et al.*, 1987). The equine isolate BEV is the best studied member of the proposed family, since it is the only torovirus so far that can be propagated in cultured cells.

Berne virus possesses a peplomer (P)-bearing envelope surrounding a tubular nucleocapsid with helical symmetry. The virion contains four polypeptides. The major protein is a phosphorylated 20K polypeptide, which has been identified as the nucleocapsid protein (N) (Horzinek *et al.*, 1985). A 22K non-glycosylated envelope protein (E) and a 37K phosphorylated matrix protein (M) are associated with the viral membrane (Horzinek *et al.*, 1985). The BEV P consists of N-glycosylated polypeptides with  $M_r$ s in the range 75K to 100K (Horzinek *et al.*, 1986).

Earlier experiments (Weiss *et al.*, 1983) have indicated that BEV possesses an RNA genome, since its growth is not inhibited by iododeoxyuridine. Viral replication is inhibited by actinomycin D (AMD),  $\alpha$ -amanitin and u.v. pre-irradiation of the cells suggesting that the expression of a cellular gene is essential for BEV multiplication (Horzinek *et al.*, 1984).

In this paper some properties of the genome of BEV are reported and the presence of five BEV-specific poly(A)-containing RNAs in infected cells is described.

### METHODS

*Cells and virus.* Embryonic mule skin (EMS) cells were propagated in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Flow Laboratories) supplemented with 10% foetal calf serum (FCS; Flow Laboratories), penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). The FCS was pretested for the absence of antibodies against BEV (Weiss *et al.*, 1983). Confluent cultures ( $10^5$  cells/cm<sup>2</sup>) were rinsed with phosphate-

buffered saline (PBS) containing 50 mg/l DEAE-dextran and infected with BEV (strain P138/72). In most experiments the inoculum was from a seed lot pretreated at an earlier passage level with 2-phenyl ethanol for inactivation of contaminating mycoplasma (Staal & Rowe, 1974). After 1 h adsorption the inoculum was removed and DMEM containing 3% FCS and antibiotics was added. Supernatants were harvested at 24 to 30 h post-infection (p.i.), the beginning of c.p.e.

Breda II virus purified from faecal material by pelleting and isopycnic centrifugation was kindly provided by G. Woode (Texas A & M University, College Station, Tx., U.S.A.). Propagation and purification of mouse hepatitis virus (MHV), a coronavirus, has been described previously (Spaan *et al.*, 1981).

Titration of infectivity, virus neutralization and ELISA methods have been described in detail (Weiss *et al.*, 1983).

*Virus purification and gradient analysis.* Supernatants of infected cultures were clarified by low-speed centrifugation. Ammonium sulphate precipitation (50% saturation) was allowed to proceed for 1 to 2 h at 4 °C. After low-speed centrifugation, the pellet was resuspended in TES buffer (10 mM-Tris-HCl pH 7.4, 1 mM-EDTA, 150 mM-NaCl), clarified again and the remaining ammonium sulphate was removed by gel filtration (Sephadex PD-10 column; Pharmacia). Linear sucrose gradients were prepared and centrifuged under equilibrium (Weiss *et al.*, 1983) or rate zonal (Horzinek *et al.*, 1985) conditions as reported previously. Preformed isokinetic sucrose gradients were used for the calculation of sedimentation coefficients as described by Van der Zeijst & Bloemers (1976); the tubes were centrifuged in SW27 or SW28.1 rotors of a Beckman L65 ultracentrifuge equipped with an  $\omega^2/dt$  integrator. After fractionation, the infectivity was determined for each fraction (Weiss *et al.*, 1983). Metabolically labelled BEV was located by liquid scintillation counting.

*Isotope labelling and isolation of genomic RNA.* EMS cell cultures were infected as described, and at 4 h p.i. the medium was replaced by phosphate-free DMEM supplemented with 2% dialysed FCS. One hour later, carrier-free [<sup>32</sup>P]orthophosphate was added, to result in a final isotope concentration of 500  $\mu$ Ci/ml. For uridine labelling, infection was performed as described above and 25 to 50  $\mu$ Ci/ml of [<sup>3</sup>H]uridine was added 4 h p.i.; material was harvested 24 h p.i. All isotope preparations were purchased from Amersham. To isolate the viral genome, gradient-purified virus was treated with 0.4% SDS, 400  $\mu$ g/ml proteinase K and 500  $\mu$ g/ml tRNA for 30 min at 37 °C followed by 30 min at 56 °C. The RNA was extracted with phenol/chloroform and ethanol-precipitated.

*RNA transfection assay.* The method has been described before (Van der Zeijst *et al.*, 1986). In short, confluent monolayers of EMS cells in 35 mm Petri dishes were rinsed with PBS and hypertonic buffer 1 (HB1; 600 mM-NaCl in 10 mM-Tris-HCl pH 7.5, containing 20  $\mu$ g/ml of polyvinyl sulphate), and then incubated for 5 min with hypertonic buffer 2 (HB2; as above, but containing 1.1 M-NaCl). The RNA preparation in 200  $\mu$ l of HB2 was pipetted onto the monolayers and incubated for 15 min; the inoculum was then removed, cells were washed once with HB1 and DMEM with 10% FCS was added. All manipulations were done at room temperature. The c.p.e. was read after 3 days of incubation. The virus specificity and single-strandedness of the transfecting RNA was demonstrated by treatment of the extracts with bovine pancreatic RNase A (5 mg/ml, in 140 mM-NaCl, 14 mM-sodium citrate and 10 mM-MgCl<sub>2</sub>) for 30 min at 30 °C.

*Poly(A) selection and agarose gel electrophoresis.* Oligo(dT)-cellulose chromatography was performed as described by Maniatis *et al.* (1982). RNA was analysed in 1% agarose gels after denaturation with glyoxal/dimethylsulphoxide (Spaan *et al.*, 1981) or formamide/formaldehyde (Meinkoth & Wahl, 1984). Gels were fixed in methanol, soaked in En<sup>3</sup>Hance (New England Nuclear), if necessary, and dried. Fluorography was performed as described by Laskey (1980).

*Filter spot hybridization.* Poly(A)-selected [poly(A)<sup>+</sup>] RNA from infected and uninfected cells was used as template for the synthesis of labelled cDNA as described before (van Berlo *et al.*, 1986). RNA was spotted onto nitrocellulose filters and RNA:DNA hybridization was performed essentially as described by van Berlo *et al.* (1986) at 52 °C.

*<sup>3</sup>H labelling and extraction of intracellular RNAs.* EMS cells grown in 35 mm tissue culture dishes were infected at a high m.o.i. (5 to 10) as described above. At 1 h p.i. the inoculum was removed and DMEM containing 3% FCS and 50  $\mu$ Ci/ml [<sup>3</sup>H]uridine was added. The cells were lysed at 13 h p.i. and RNA was extracted as described by Spaan *et al.* (1981). When AMD was used to inhibit host cellular RNA synthesis, the drug was added late after infection (8 h p.i.) at a concentration of 1  $\mu$ g/ml; 1 h later, the cells were labelled by adding 50  $\mu$ Ci/ml [<sup>3</sup>H]uridine, and at 13 h p.i. the RNA was extracted.

*Measurement of [<sup>3</sup>H]uridine incorporation into RNA.* Samples were spotted on Whatman 3MM filter paper and TCA-precipitated. Radioactivity was measured by liquid scintillation counting. After electrophoresis, RNA bands were excised from the dried agarose gels, soaked in scintillation fluid and counted.

*Isolation of mRNAs from infected cells.* At 13 h p.i. total RNA was isolated from  $2.7 \times 10^8$  BEV-infected (m.o.i. approx. 7) EMS cells grown in plastic roller bottles (Costar; surface area 900 cm<sup>2</sup>). Cell lysis and RNA extraction were performed as described by Spaan *et al.* (1981) and 300  $\mu$ g poly(A)<sup>+</sup> RNA was recovered after oligo(dT)-cellulose column chromatography and ethanol precipitation. Of this material 200  $\mu$ g was mixed with <sup>3</sup>H-labelled poly(A)<sup>+</sup> RNA from  $2 \times 10^7$  BEV-infected EMS cells; this mixture was separated on a 5 to 29% (w/w) isokinetic sucrose gradient by ultracentrifugation for 4 h at 41 000 r.p.m. ( $\omega^2 t = 2.7 \times 10^{11}$  rad<sup>2</sup>/s) in a Beckman SW41 rotor

(Van der Zeijst & Bloemers, 1976). Sucrose solutions and RNA samples were prepared as described by Jacobs *et al.* (1986). The RNA in the resulting 36 fractions (330  $\mu$ l each) was recovered by ethanol precipitation and used for analysis in agarose gels and *in vitro* translation.

*In vitro translation of mRNAs.* One-third of the precipitate from each gradient fraction (1  $\mu$ l) or 5  $\mu$ g total polyadenylated RNA were translated by adding 10  $\mu$ l of a rabbit reticulocyte lysate (N.90; Amersham) and 1  $\mu$ l of [<sup>35</sup>S]methionine (15  $\mu$ Ci). After 60 min of incubation at 30 °C the reaction mixtures were cooled on ice, diluted twofold in TES buffer containing 4 mM-PMSF and analysed by SDS-PAGE, either directly or after immunoprecipitation (see below).

*Radioactive labelling of virion proteins.* Virus was labelled with [<sup>35</sup>S]methionine by incubating infected cell cultures from 9 to 12 h p.i. in methionine-deficient DMEM, supplemented with 3% dialysed FCS and 50  $\mu$ Ci/ml [<sup>35</sup>S]methionine. Virions were isolated from the cell culture medium by ultracentrifugation at 200000 *g* at 4 °C for 2 h. Pellets from infected and mock-infected culture supernatants were dissolved in electrophoresis sample buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 10% glycerol, 2% w/v SDS, 5% 2-mercaptoethanol, 0.001% w/v bromophenol blue) and used as viral protein markers in SDS-PAGE.

*Immune sera and monoclonal antibodies.* A field serum from a horse with BEV neutralizing antibodies was used in radioimmunoprecipitations. In addition, monoclonal antibodies (MAbs) directed against the BEV N and P proteins (MAbs 1F1 and 6B10, respectively; B. Kaeffer *et al.*, unpublished data) were used.

*Radioimmunoprecipitation (RIP) and SDS-PAGE.* Viral proteins were precipitated by adding 7.5  $\mu$ l aliquots of the anti-BEV horse serum or 5  $\mu$ l volumes of anti-BEV MAB ascites fluid to 10  $\mu$ l portions of diluted translation mixtures. The mixtures were diluted in TES buffer containing 2 mM-PMSF to result in final volumes of 75  $\mu$ l. After 15 min of incubation at 37 °C, 50  $\mu$ l of a 10% (w/v) suspension of formalin-treated *Staphylococcus aureus* cells in RIP buffer (10 mM-Tris-HCl pH 7.4, 1% w/v sodium deoxycholate, 1% NP40 and 150 mM-NaCl) was added and incubation was continued for another 15 min at 37 °C. The *S. aureus* cells with the adsorbed immune complexes were centrifuged down, washed three times in 0.5 ml RIP buffer and the final pellet was dissolved in 35  $\mu$ l of electrophoresis sample buffer. After removal of the *S. aureus* by centrifugation, samples were analysed by SDS-PAGE on gels ranging from 7.5 to 20% in polyacrylamide concentration. Fluorography was performed as described by Laskey (1980).

## RESULTS

### *Purification of virions and genome isolation*

In view of the heterogeneity in density of BEV-specific structures encountered in the supernatants of infected cells (Horzinek *et al.*, 1985), sedimentation experiments were performed in sucrose gradients. Berne virus labelled with [<sup>32</sup>P]orthophosphate or [<sup>3</sup>H]uridine was partly purified from infectious culture media and analysed in isokinetic sucrose gradients. Peaks of TCA-precipitable radioactivity were observed at positions of 380S and 50S. No labelled sedimentable material was seen in mock-infected EMS culture supernatants (data not shown). The bulk of viral infectivity and ELISA activity sedimented at a velocity corresponding to 380S (data not shown). When run in an equilibrium centrifugation, the 380S particles banded at virion density (1.16 to 1.17 g/ml; Weiss *et al.*, 1983).

Further experiments were focused on the 380S material. The RNA from this peak was extracted and analysed by agarose gel electrophoresis. A single RNA species was detected in the material containing the 380S particles (Fig. 1*a*).

### *Infectivity and polarity of the BEV genome*

A transfection assay using purified genomic RNA can be used to discriminate between positive- and negative-stranded RNA viruses. Extraction using the proteinase K/SDS/phenol method was performed on a virus preparation containing about 10<sup>10</sup> TCID of BEV. Dilutions of the RNA were transfected into EMS cell monolayers using hypertonic pretreatment. Undiluted material was digested with RNase A before transfection. After incubation of the cultures for 3 days, complete c.p.e. had developed in the preparations diluted fivefold and 25-fold, with c.p.e. beginning in the 1:125 dilution. Cytopathic changes were absent in the RNase-treated preparation. Identification of the virus released from cells transfected with the 1:125 dilution of BEV RNA was done using a neutralization assay. A neutralization index of 4.2 was found using a 1:50 dilution of a horse serum that had been selected for its antibody content.

The BEV genome was found to be polyadenylated by using oligo(dT)-cellulose column chromatography. The RNA was extracted from [<sup>3</sup>H]uridine-labelled BEV and the column

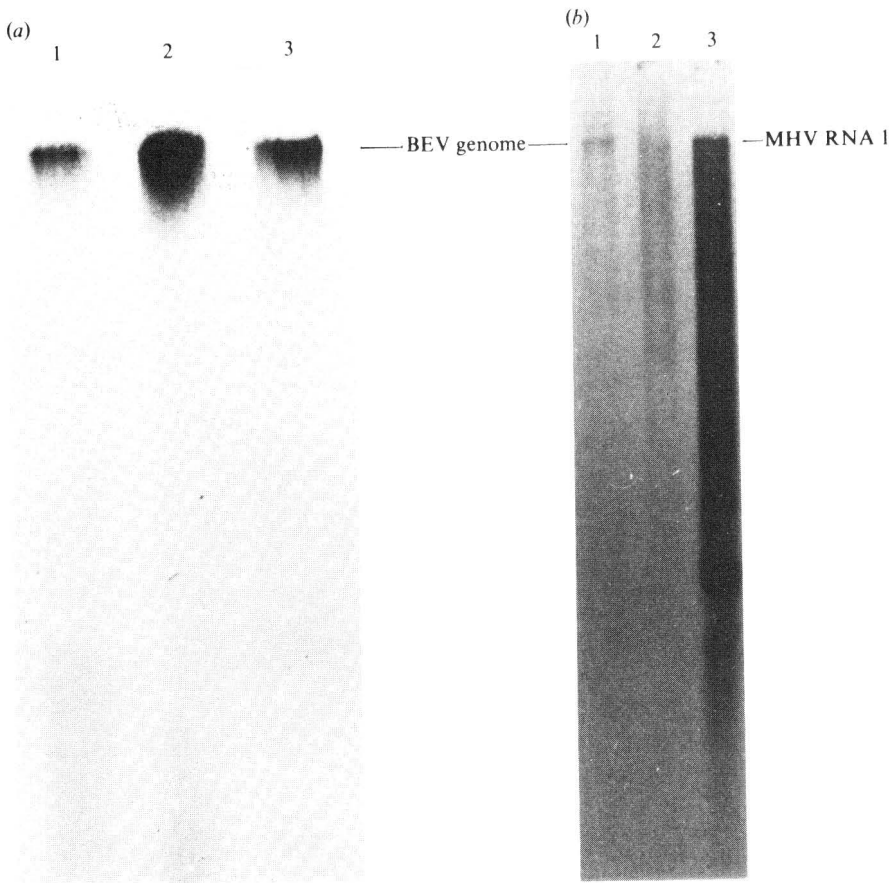


Fig. 1. (a) Agarose-urea gel electrophoresis of  $^{32}\text{P}$ -labelled BEV RNA; nucleic acid was extracted from three consecutive fractions of the 380S peak of an isokinetic sucrose gradient. (b) RNA electrophoretic patterns from an affinity chromatography experiment using an oligo(dT)-cellulose column. BEV genomic RNA (lane 1) eluted from the column and unbound flowthrough material (lane 2) is shown; note that the degradation products of BEV RNA did not bind to oligo(dT). Lane 3 shows intracellular RNAs of MHV included for comparison.

flowthrough and eluate were analysed by agarose gel electrophoresis, using MHV strain A59 intracellular RNAs as  $M_r$  markers. As depicted in Fig. 1(b), genomic RNA of BEV was bound to the column. The BEV genome comigrated with MHV intracellular RNA 1; since large RNA species are not resolved in the top (non-linear) portion of agarose gels (Lehrach *et al.*, 1977), we refrain from giving an exact figure for the genome length but estimate it to be 20 kb or longer.

To compare genome and messenger RNA polarity, filter hybridization experiments were performed using  $^{32}\text{P}$ -labelled cDNA probes prepared against poly(A)<sup>+</sup> RNA from BEV-infected cells. Hybridization occurred with RNA extracted from purified BEV (Fig. 2, A3, B3), and with poly(A)<sup>+</sup> RNA from infected cells (undiluted,  $10^{-1}$ ,  $10^{-2}$ ) serving as positive controls (Fig. 2, A1, B1, C1). No reaction was encountered with poly(A)<sup>+</sup> RNA from mock-infected cells diluted  $10^{-1}$  or more (Fig. 2, B2, C2); a faint signal occurred with the undiluted material (Fig. 2, A2). A distinct spot was detected when RNA of an antigenically related torovirus (Breda II; Woode *et al.*, 1982) was included (Fig. 2, B4).

#### *Identification of Berne virus-induced mRNAs*

In order to identify virus-specific intracellular RNA, BEV-infected EMS cells were labelled with [ $^3\text{H}$ ]uridine from 1 to 13 h p.i. Pulse labelling experiments had indicated that viral RNA synthesis reached its maximum between 9 and 13 h p.i. (data not shown). Agarose gel electrophoresis of total RNA demonstrated the presence in infected cells of five major RNA

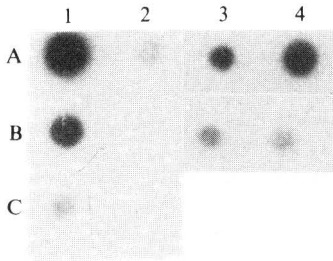


Fig. 2. Filter spot hybridization using a cDNA probe prepared against poly(A)<sup>+</sup> RNA extracted from BEV-infected EMS cells. The preparations spotted onto nitrocellulose were: poly(A)<sup>+</sup> RNA from BEV-infected cells, undiluted (A1) and at dilutions  $10^{-1}$  (B1) and  $10^{-2}$  (C1); as above, from mock-infected cells (A2, B2, C2); RNA extracted from purified BEV undiluted (A3) and at a 10-fold dilution (B3); genomic RNA from Breda II virus (B4); polyadenylic acid served as a control (A4).

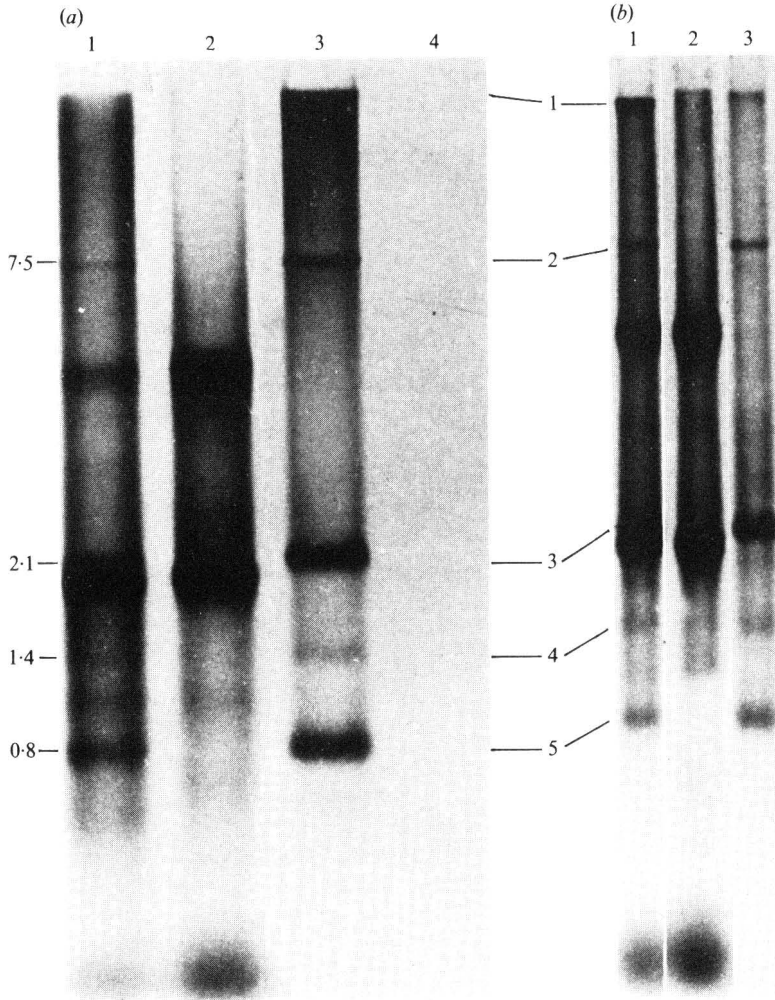


Fig. 3. Electrophoretic analysis of RNA from BEV-infected and mock-infected EMS cells. Denatured [<sup>3</sup>H]uridine-labelled RNA was analysed in 1% agarose gels. BEV-specific RNAs are numbered 1 to 5 (see Table 1), RNA length is given in kb. (a) Total cellular RNA from infected cells (labelled from 1 to 13 h p.i.) was run in lane 1, RNA from mock-infected cells in lane 2. Lanes 3 and 4 show RNA from infected and uninfected cells, labelled from 9 to 13 h p.i. in the presence of AMD. MHV mRNAs and eukaryotic rRNAs were used as markers to calculate BEV RNA lengths. (b) Poly(A)<sup>+</sup> RNA was prepared using oligo(dT)-cellulose column chromatography. Total cellular RNA from infected cells was run in lane 1; lane 2 shows the flowthrough material and lane 3 the poly(A)<sup>+</sup> RNA.

species that were absent from uninfected cells (Fig. 3a). To demonstrate the viral origin of these RNA species, we used AMD, an inhibitor of DNA-dependent RNA transcription. Although this drug is known to interfere with BEV replication, its influence decreases when added late after infection (Horzinek *et al.*, 1984). We labelled cells with [<sup>3</sup>H]uridine from 9 to 13 h p.i. after

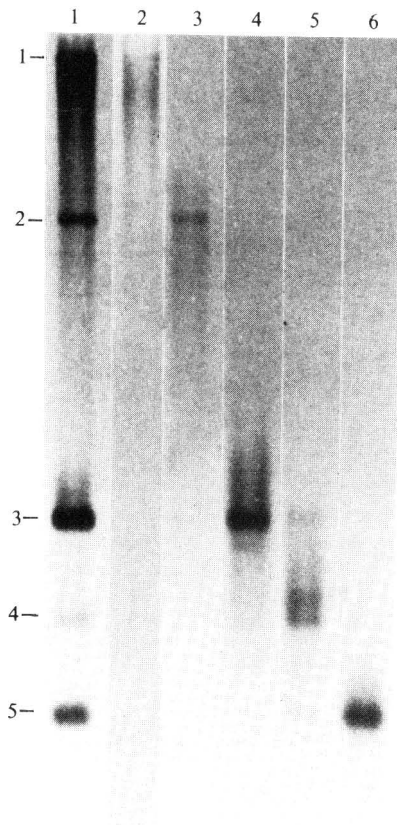


Fig. 4. Fractionation of poly(A)<sup>+</sup> RNA from BEV-infected EMS cells in an isokinetic sucrose gradient. The RNA content of the gradient fractions was analysed in a 1% agarose gel. <sup>3</sup>H-labelled RNA from AMD-treated cells was run in lane 1 as a viral RNA marker. Lanes 2 to 6 show the RNA recovered from fractions 4, 20, 27, 29 and 31, containing the largest amounts of BEV RNA 1, 2, 3, 4 and 5, respectively.

Table 1. *Characteristics of BEV-specific intracellular RNAs*

RNA	$M_r$ ( $\times 10^{-6}$ ) <sup>*</sup>	RNA length (kb) <sup>*</sup>	Relative molarity (%) <sup>†</sup>	Sedimentation coefficient (S) <sup>‡</sup>
1	>6.7	>20	2.2	65
2	2.53	7.5	3.4	33
3	0.71	2.1	29.5	19
4	0.47	1.4	12.7	15
5	0.27	0.8	52.2	11

\* Calculated from agarose gels, using eukaryotic rRNAs and MHV-specific mRNAs as markers.

† Intracellular RNA was labelled with [<sup>3</sup>H]uridine from 9 to 13 h p.i. in the presence of AMD. RNA bands were excised from agarose gels and counted. Relative molarities were calculated using the  $M_r$  data from this table.

‡ As estimated from the sedimentation behaviour of poly(A)<sup>+</sup> selected RNAs in a 5 to 29% (w/w) isokinetic sucrose gradient (Van der Zeijst & Bloemers, 1976).

addition of 1  $\mu$ g/ml AMD at 8 h p.i. It can be concluded from Fig. 3(a) lane 3 that in the presence of the drug the same five RNAs were synthesized in infected cells.

Using cellular 18S and 28S rRNAs and the mRNAs of MHV (strain A59) as markers, the lengths of the BEV RNAs were determined. Recent sequencing data from our group were used to calculate the  $M_r$  of the MHV intracellular RNAs (W. Luytjes & P. J. Bredenbeek, personal communication). The BEV RNAs were calculated to be 7.5 (RNA 2), 2.1 (RNA 3), 1.4 (RNA 4), 0.8 (RNA 5) and at least 20 (RNA 1) kb in length (Table 1). To quantify the relative amounts of

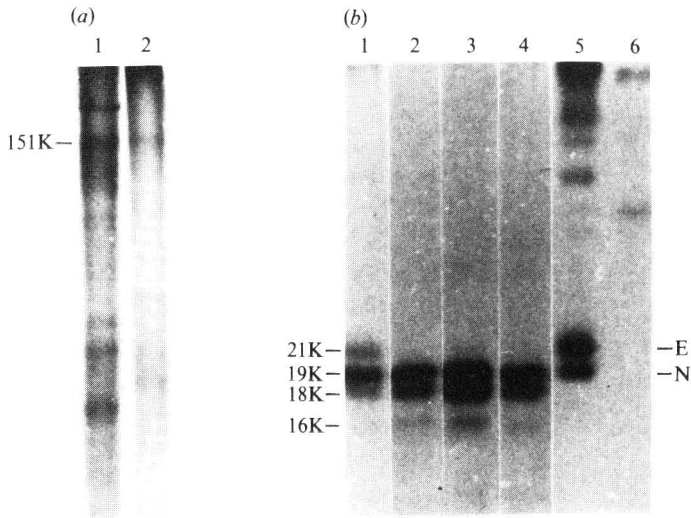


Fig. 5. SDS-PAGE analysis of *in vitro* translation products of fractionated poly(A)<sup>+</sup> RNA from BEV-infected EMS cells. (a) Lane 1 shows a direct analysis (10% gel) of translation products when RNA from gradient fraction 20 (containing the RNA 2 peak) was added to the system. In lane 2 the same translation mixture was analysed, but now after precipitation using a horse immune serum. (b) Using the same serum the products of translation mixtures programmed with RNA from gradient fractions 27 (RNA 3; lane 1), 29 (RNA 4; lane 2) and 31 (RNA 5; lane 3) were precipitated and analysed on a 20% acrylamide gel. Lane 4 also shows a precipitate of the products of fraction 31, but now immunoprecipitation was performed using the 1F1 MAb, directed against the BEV N protein. <sup>35</sup>S-labelled virions were isolated from the medium of infected EMS cells, denatured and used as markers for BEV structural proteins (lane 5). Lane 6 shows the result when the medium of a mock-infected cell culture was used.

RNA synthesized between 9 and 13 h p.i. in the presence of AMD, individual bands were excised from the gel and counted; the resulting molarities are shown in Table 1.

The five virus-specific RNAs were further characterized by oligo(dT)-cellulose column chromatography of <sup>3</sup>H-labelled total RNA from infected cells (which had not been treated with AMD). All five RNAs were found to bind to the column (Fig. 3b).

#### *Isolation and fractionation of virus-specific intracellular RNAs*

To purify BEV-specific intracellular RNAs for *in vitro* translation, RNA was isolated from BEV-infected EMS cells by phenol extraction and ethanol precipitation. Poly(A)<sup>+</sup> RNA was prepared by oligo(dT)-cellulose column chromatography. Sixteen percent of the total RNA was bound to the column, while only 5% of the material from mock-infected cells was retained.

A mixture of <sup>3</sup>H-labelled and unlabelled poly(A)<sup>+</sup> material from infected cells was layered on top of an isokinetic sucrose gradient and the RNA was fractionated after ultracentrifugation. The RNA content of each fraction was analysed by agarose gel electrophoresis using <sup>3</sup>H-labelled RNA from infected, AMD-treated cells as markers. Peak amounts of the BEV-specific RNAs 1 to 5 were recovered from fractions 4, 20, 27, 29 and 31, respectively (Fig. 4). The sedimentation coefficients calculated for these RNA species are listed in Table 1.

#### *Translation of individual mRNAs of Berne virus*

Samples enriched for individual BEV RNAs were used for *in vitro* translation to establish the coding assignments for the five viral RNA species. No protein product could be detected after translation of the RNA from fraction 4 (containing the largest quantity of RNA 1). When RNA 2-containing fractions were translated, a 151K polypeptide product appeared (Fig. 5a, lane 1). Its highest level of synthesis coincided with the maximum amount of RNA 2 (fraction 20). However, this protein was recognized only poorly in immunoprecipitation using a BEV-specific horse serum (Fig. 5a, lane 2). The results were even less conclusive when the precipitation was

performed using a neutralizing MAb (6B10), which recognizes the BEV P proteins and their intracellular precursor (data not shown).

Due to the small differences in size between BEV RNAs 3, 4 and 5, considerable cross-contamination remained after separation in a sucrose gradient (Fig. 4, lanes 4, 5 and 6). Nevertheless, peaks of translation were observed for the 21K product in fraction 27 (corresponding to RNA 3) and for the 19K, 18K and 16K polypeptides in fraction 31 (containing the largest amount of RNA 5). No protein product(s) could be assigned to fraction 29 (the peak of RNA 4). The 21K and 19K products co-electrophoresed with the two most prevalent viral proteins in both virions and BEV-infected cells, the unglycosylated envelope protein and the BEV nucleocapsid protein, respectively (Fig. 5*b*, lanes 1 to 6). In immunoprecipitations, the 21K, 19K, 18K and 16K translation products were recognized by the horse serum (Fig. 5*b*, lanes 1 to 3). In addition, the 19K, 18K and 16K polypeptides could be precipitated using the 1F1 MAb, directed against the BEV nucleocapsid protein (Fig. 5*b*, lane 4).

#### DISCUSSION

In previous studies we have shown that virus-specific material released from EMS cells after infection with BEV consists of two major density classes: the infectious virion (1.16 to 1.17 g/ml in sucrose; Weiss *et al.*, 1983) and peaks of virus-specific activities with densities of 1.11 g/ml and lower (Horzinek *et al.*, 1985). In the present study sedimentation heterogeneity was observed, which resulted in the identification of virus-specific 380S and 50S particles. The 380S material contains the bulk of infectious virions. The 50S material, when centrifuged in an equilibrium gradient, banded at a density of 1.11 g/ml. Purification of the 380S particles was essential for the characterization of the BEV genomic RNA.

A positive polarity for the torovirus genome was established with the aid of transfection and filter hybridization experiments. Polyadenylation was also found, which is another indication of positive polarity. Lack of polyadenylation, however, does not exclude it; flavi- and pestiviruses possess genomes of messenger polarity without poly(A) tracts (Schlesinger, 1980; Renard *et al.*, 1985).

In this paper the presence of at least five virus-specific RNA species in BEV-infected cells is demonstrated. The fact that these RNAs are polyadenylated suggests that they can function as mRNAs. Identification of the translation products of RNAs 3 and 5 as the viral E and N proteins is direct evidence for this assumption and demonstrates a correlation in relative abundance between viral RNAs and proteins. The presence in infected cells of smaller polypeptides related to the BEV N protein has been reported previously (Horzinek *et al.*, 1985). The 18K and 16K translation products can therefore be considered as premature termination products of N protein synthesis or its proteolytic fragments. This phenomenon resembles the degradation during immunoprecipitation of the MHV N protein translated *in vitro*, as reported for the strains A59 and JHM (Rottier *et al.*, 1981; Siddell *et al.*, 1980).

The identification of the 151K translation product of RNA 2 as virus-specific is not unambiguous. However, in tunicamycin-treated infected cells a protein of similar size is synthesized. This polypeptide has been identified as the unglycosylated form of the 200K intracellular precursor of the BEV peplomer proteins (Horzinek *et al.*, 1986). For the moment the recognition of the 151K product by horse immune serum, though poorly, will have to suffice as evidence. Identification of this product may have been hampered by the low amount of RNA 2 in translation lysates and/or poor antigenicity of the unglycosylated polypeptide.

The appearance of multiple subgenomic RNAs in infected cells is not unusual for positive-stranded RNA viruses. Nested sets of viral mRNAs, with 3' coterminal ends and expression of only the 5'-terminal non-overlapping region, have been identified in cells infected with various coronaviruses (for a review, see Siddell *et al.*, 1982). In the togavirus family, equine arteritis virus also produces a 3' nested set (W. J. M. Spaan, unpublished data); the alphaviruses synthesize RNA of genome size plus one subgenomic mRNA, which again represents the 3'-terminal part of the genome (for a review, see Strauss & Strauss, 1983). Several positive-stranded plant viruses have also been reported to produce subgenomic mRNAs during their



multiplication (Goelet & Karn, 1982; Joshi & Haenni, 1984). It will be interesting to see whether the BEV RNAs contain common sequences and in which way the subgenomic species are generated. The sizes of the translation products of RNAs 2, 3 and 5 are in good agreement with the theoretical non-overlapping coding capacities of a 3' coterminal nested set (26K, 23K and 178K, respectively).

A number of similarities and differences between toroviruses and coronaviruses have been described. They resemble each other in having peplomer-bearing envelopes, a positive-stranded RNA genome of comparable size and multiple subgenomic mRNAs; however, the details of virion morphology, morphopoiesis and protein composition, as well as the sensitivity to AMD, discriminate these two families of viruses. At the molecular level, the sizes of the nucleocapsid RNAs and their translation products are different for toroviruses and coronaviruses. An 0.8 kb RNA encodes the 19K BEV N protein, whereas the nucleocapsid protein of coronaviruses has a characteristic size of 45K to 55K and is translated from a much larger RNA species (e.g. 1.8 kb for MHV A59). To study the replication strategy and genome organization, the molecular cloning of BEV RNA is in progress in our laboratory.

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