The Nucleocapsid of Berne Virus

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SUMMARY

In Berne virus-infected cells and in gradient-purified virions two major proteins with mol. wt. of 20K and 22K were detected. The 22K species is thought to represent the main envelope polypeptide; in infectious culture media it was present in a low density substructure which could be quantitatively converted into slowly sedimenting material by detergent treatment. The 20K polypeptide (accounting for about 80% of the ^1^4C-amino acid label in the virion) was phosphorylated, occurred in an intracellular substructure of higher density than the virion (ρ = 1.36 g/ml in CsCl) and was the only viral protein possessing RNA-binding properties; it was recognized preferentially by heterologous animal sera in immune precipitation. The 20K species is therefore identified as the main capsid protein. Two additional polypeptides (19K and 17K) were regularly detected in extracts of infected cells; they appeared to share oligopeptides with the 20K protein and are interpreted as being proteolytic cleavage products. The nucleocapsid of Berne virus was visualized after ether treatment as a flexible bacilliform structure with conspicuous transverse striation. Demonstration of a 20K nucleocapsid protein further supports the authors' proposal that Berne virus is a representative of a new family of enveloped RNA viruses (Toroviridae).

INTRODUCTION

The purification and partial characterization of a new enveloped RNA virus isolated from a horse during routine diagnostic work in Berne, Switzerland, has been reported (Weiss et al., 1983). 'Berne virus' measures 120 to 140 nm at its largest diameter and consists of a peplomer-bearing envelope and an elongated core which is bent into an open torus within the membrane. The core is tubular in appearance and has a morphology indicative of helical symmetry; it is considered to represent the viral nucleocapsid. The virus possesses an RNA genome since its growth is not affected by DNA nucleotide analogues; actinomycin D and α-amanitin on the other hand do inhibit replication as does u.v. pre-irradiation of the cells (Horzinek et al., 1984).

Berne virus is serologically unrelated to known equine viruses and to representatives of the three main antigenic clusters of coronaviruses (infectious bronchitis, mouse hepatitis and transmissible gastroenteritis viruses) which it resembles superficially in negatively stained preparations. A serological relationship has been discovered, however, with the enteropathogenic Breda viruses isolated from calves (Woode et al., 1982; Weiss et al., 1983), Lyon-4 virus of bovines (Moussa et al., 1983) and with particles encountered in the stools of humans with gastroenteritis (Beards et al., 1984).

In the present paper, experiments to identify and localize the polypeptides of Berne virus are reported. The finding of a major nucleoprotein of 20K further supports our suggestion (Horzinek, 1984) that the Berne/Breda group of viruses occupies a unique position in viral taxonomy and should be assigned a family status (Horzinek & Weiss, 1984).
METHODS

Cell culture and propagation of virus. Embryonic mule skin (EMS) cells were propagated in Dulbecco's modification of Eagle's MEM with Earle's salts (DMEM), supplemented with 2 to 10% foetal calf serum (FCS), non-essential amino acids (1%), l-glutamine (200 mM), sodium bicarbonate and antibiotics. The calf serum was pretreated by neutralization assay for the absence of antibody against Berne virus. Subconfluent EMS cell cultures were rinsed with phosphate-buffered saline containing 50 mg/ml DEAE-dextran and inoculated with the P138/72 strain of Berne virus at a multiplicity of about 1. Supernatants and cells were harvested separately at 18 to 24 h post-infection.

Isotope labelling. At 4 h post-infection, the medium was removed. In the case of $[^3]$H]methionine labelling, 2 ml methionine-free MEM supplemented with 2% FCS and containing 110 μCi of the labelled amino acid were used per 25 cm$^2$ plastic bottle (Costar). For carbon labelling, 30 μCi of a $[^14]$C]protein hydrolysate in DMEM were used. Inorganic phosphate labelling was performed in phosphate-free, HEPES-buffered medium supplemented with 2% dialysed FCS, using 0.5 mCi/ml carrier-free $^{32}$PO$_4$ per 10 cm$^2$ plastic Petri dish (Costar). Isotopes were purchased from Amersham. After the labelling period, the supernatants were clarified by low-speed centrifugation and stored at $-20\,^\circ$C. The monolayers were rinsed three times with TES buffer (0-02 M-Tris-HCl pH 7.4, 1 mM-sodium EDTA, 0.1 M-NaCl) and lysed with 0.5% Triton X-100 and 0.5% 1,5-naphthalenedisulphonic acid, disodium salt (Eastman Kodak) in TES (lysis buffer). After centrifugation for 5 min at 10000 g, the supernatants were analysed directly or after immune precipitation.

The methods of immune precipitation and SDS–PAGE have been described previously (Horzinek et al., 1982); in some cases, immune complexes were aggregated using a second antibody instead of Protein A from Staphylococcus aureus. Proteins were visualized by fluorography on preflashed Fuji RX film at $-70\,^\circ$C (Laskey & Mills, 1975). For this purpose the gel slabs had been soaked in two changes of DMSO (J. F. Baker Chem., Deventer, The Netherlands) for 30 min each, followed by overnight incubation in $20\%$ (w/w) PPO (Merck) in DMSO. They were subsequently incubated twice for 30 min in tap water and dried onto a sheet of filter paper under vacuum and heating.

Gradient analysis. Linear sucrose gradients were prepared and centrifuged under equilibrium conditions as described previously (Weiss et al., 1983). For the isolation of nucleocapsids from infected cells, CsCl was dissolved in TES buffer to give a density of 1.34 g/ml and 5 ml of the solution was mixed with 200 μl of the cell lysate. After 24 h of centrifugation at 40000 r.p.m. in a Spinco SW50.1 rotor (130000g), the self-forming gradient was fractionated and the densities calculated from the refractive indices measured. Rate zonal centrifugations were performed in 10 to 40% linear sucrose gradients spun for 16 h at 200000 r.p.m. in a Spinco SW27.1 rotor.

Identification of RNA-binding proteins. A modification of the method described by Bowen et al. (1980) for the detection of DNA-binding proteins by blotting was employed. In short, lysates from Berne virus-infected and mock-infected EMS cells were incubated with a rabbit antiserum which had been shown by radiimmunoprecipitation (RIP) to recognize preferentially the 20K protein (see Fig. 6, lanes 11 and 12). The immune complexes were precipitated using a goat anti-rabbit IgG serum (Miles-Yeda, Rehovot, Israel) and separated on 15% SDS-polyacrylamide gels. Subsequently, the proteins were renatured by gentle agitation for 3 h in a urea-containing buffer (50 mM-NaCl, 2 mM-sodium EDTA, 4 M-urea, 0.1 mM-dithiothreitol, 10 mM-Tris–HCl pH 7.0). Transfer of the proteins to nitrocellulose filters (BA-85, Schleicher & Schuell) sandwiching the gel was allowed to proceed by diffusion at room temperature. The compression device described by Bowen et al. (1980) was used; the apparatus remained submerged for 40 h in a buffer containing 50 mM-NaCl, 2 mM-sodium EDTA, 0.1 mM-dithiothreitol, 10 mM-Tris–HCl pH 7.0. The effectiveness of the procedure was monitored by transferring $[^3]$H]methionine-labelled immune complexes, from Berne virus-infected and mock-infected cell extracts, from gels to nitrocellulose filters. The proteins were visualized by fluorography after the filters had been sprayed with 20% PPO in toluene and air-dried.

After transfer, the filters were soaked for 90 min in binding buffer (0-05 M-NaCl, 1 mM-sodium EDTA, 10 mM-Tris–HCl pH 7.0, 0.02% bovine serum albumin, 0.02% Ficol, 0.02% polyvinylpyrrolidone) and then incubated with the radioactive probe (2 × 10$^6$ c.p.m./10 ml) for 60 min in a sealed plastic pouch (Seal-a-Meal, Daze). Unbound material was removed by five rinses with binding buffer for 90 min at 50 °C in the presence of 50 mM-Na$_2$CO$_3$ (about pH 12). After adjustment to pH 7.6 the fragments were ethanol-precipitated, the pellet was resuspended in 10 μl distilled water and labelling performed using 80 μCi $[^32]$P]ATP in the presence of 4.5 U T4 polynucleotide kinase (Boehringer Mannheim). Incubation was for 30 min at 37 °C in 25 μl of a buffer containing 17 mM-Tris–HCl pH 7.6, 5 mM-MgCl$_2$, 5 mM-dithiothreitol. Unincorporated radioactivity was removed by gel filtration on a 1 ml spin column (Maniatis et al., 1982) of Sephadex G50 (medium; Pharmacia) in TES buffer pH 8.0. Fluorographic detection of RNA-binding proteins was performed on preflashed Fuji RX film using an intensifying screen as described above.

Limited proteolysis. From a dried 20% PAGE gel on which a lysate of Berne virus-infected cells had been analysed, the 22K, 20K, 19K and 17K bands were cut out after having been localized by fluorography. The pieces
were soaked overnight in a buffer containing 0.125 M-Tris-HCl pH 6.8, 0.1 % SDS, 1 % dithiothreitol, 1 mM-sodium EDTA, 0.001 % bromphenol blue (elution buffer). The gel fragments were then inserted into the slots of a 5 % stacking gel on top of a 20 % running gel; they were covered with elution buffer containing 20 % glycerol. The same buffer mixture (20 µl) containing 1 µg S. aureus V8 protease (Miles-Yeda) was layered on top and the current switched on until the dye front had run to about 1 mm from the stacking gel/running gel interface. Electrophoresis was discontinued for 30 min to allow enzyme digestion and resumed under the usual conditions. Heated (1 min at 100 °C) and unheated undigested lysate material and a radioiodinated low mol. wt. marker set (28.7K to 12.5K) were analysed in parallel.

**Electron microscopy.** Nucleocapsids were liberated from semi-purified virus (ammonium sulphate precipitation, centrifugation into a 20/40 % sucrose interface) dried onto Formvar-coated, carbon-stabilized grids by short immersion (15 to 60 s) in diethyl ether. Staining was performed using 1 % unbuffered uranyl acetate in water. The preparations were examined immediately in a Philips EM 300 electron microscope operating at 80 kV.

**RESULTS**

**Density gradient analysis**

EMS cells were infected with Berne virus or mock-infected, respectively, and [35S]methionine label was added 4 h post-infection. At 21 h post-infection, the media were harvested and ammonium sulphate precipitation was performed as described previously (Weiss *et al.*, 1983). Sixty-four % of the radioactivity was recovered in the sediment from the infected cells, whereas 51 % was precipitable from the mock-infected medium. Upon centrifugation in a linear 10 to 50 % sucrose gradient, the radioactivity present in the mock preparation had hardly moved from the meniscus (Fig. 1 a). In the gradient containing material from infected cultures, three main peaks were detected, with apparent densities of 1.16 (fraction 7, the virion peak), 1.11 (fraction 14) and 1.07 g/ml (fraction 19). PAGE was performed with material from these and two fractions between the peaks, fractions 12 and 16; Fig. 1 (c) shows the two major proteins of Berne virus (Horzinek *et al.*, 1984) and a conspicuous increase in the 20K/22K signal ratio with greater buoyant density of the structure. In the virion peak, the 20K protein was most abundant; a 17K polypeptide was observed in this fraction only. Additional proteins of 37K and 80K to 120K were noted at the virion density.

In the 1.07 g/ml peak (fraction 19), the 22K protein was most prominent with the 37K species also appearing. No signal was observed at the 20K position. Further analysis of this fraction by rate zonal centrifugation showed that the radioactivity sedimenting at about virion velocity was quantitatively converted to slowly sedimenting material by treatment with 1 % Triton X-100. Detergent treatment of virions (fraction 7) resulted in the appearance of a similar, slowly sedimenting fraction in addition to a peak of slightly less than virion velocity. In Fig. 1 (b) the position of virions in the gradient is indicated (arrow); for better dispersion, the preparation had been pretreated with 0.2 % sodium deoxycholate, which has no effect on viral infectivity (Weiss & Horzinek, 1985).

In another experiment, Triton X-100 was added to the supernatant of a Berne virus-infected, labelled EMS cell culture to a final concentration of 0.5 % and layered on top of a 37 % sucrose cushion (corresponding to the virion density of 1.16 g/ml). After centrifugation at 100000 g for 5 h, the top material was saved, the sucrose cushion discarded and the pellet resuspended in TES. Distribution of TCA-precipitable counts in the top and pellet preparations was approximately 97 % and 3 % respectively. In the top layer, the 22K polypeptide was the predominant viral structure; no signal could be detected in the resuspended pellet (results not shown). Therefore, RIPs using a rabbit anti-Berne virus immune serum were performed on both preparations which resulted in a recovery of about 10 % of the label in the supernatant and about 66 % in the pellet fraction. PAGE of the immune precipitates revealed the 22K and 20K polypeptides in the supernatant fraction (Fig. 2, lane 2) whereas the pellet contained the 20K species only (lane 3). The 20K protein appears to be phosphorylated (although to a lesser extent than the 37K protein) as shown in lanes 4 and 5 of Fig. 2. Its prevalence in the virion was determined by measuring the distribution of radioactivity in 14C-amino acid-labelled, gradient-purified virus (lane 6). The 20K band contained 79 % of virus-specific label whereas in the 80K to 120K, 37K and 22K bands values of 3 %, 4 % and 14 %, respectively, were found; no signal
Fig. 1. Centrifugation in sucrose gradients of \[^{35}S\]methionine-labelled Berne virus concentrated by ammonium sulphate precipitation. The graph (a) shows the radioactivity distribution in an isodensity gradient of preparations from infected (●) and mock-infected cells (◯). The autoradiograph (c) represents the polypeptide patterns of fractions 7 (virion density: 1.16 g/ml), 12, 14 (1.11 g/ml), 16 and 19 (1.07 g/ml), indicated by arrows on (a). Rate zonal centrifugation was performed (b) with material from fraction 19 of the isodensity gradient either directly (□) or after treatment with Triton X-100 (■). The position of untreated virions is indicated (400S; arrow); detergent-treated virion material was included for comparison (●). Sedimentation was from right to left.

Fig. 2. PAGE of the radioimmunoprecipitates of the supernatant (lane 2) and the pellet fraction (lane 3) of a \[^{35}S\]methionine-labelled lysate of Berne virus-infected cells after centrifugation (100000 g, 5 h) through a 37% sucrose cushion; lane 1 shows the immune precipitate of the unfractionated lysate. In lane 4, a \(^{32}\)PO\(_4\)-labelled preparation was analysed and lane 5 shows the corresponding mock-infected sample. To demonstrate the predominance of the 20K protein in purified virions, a \(^{14}\)C-amino acid-labelled preparation was analysed in lane 6.
could be detected in the position of the 17K polypeptide. By taking into account the differences in mol. wt., the relative abundance of the polypeptides was calculated as 84% (20K), 13% (22K), 2% (37K) and 1% (80K to 120K range).

Since nucleocapsid-like strands have been encountered in the cytoplasm of Berne virus-infected cells (Weiss et al., 1983), extracts were thought to be a richer source of capsid protein. Isopycnic centrifugation of a labelled Triton X-100 cellular lysate in a CsCl gradient resulted in accumulation of the 22K protein at the meniscus whereas both the 20K and 17K polypeptides penetrated into the gradient to a maximum density of 1.36 g/ml; no corresponding bands were visible in the mock-infected preparation (Fig. 3).

**Limited proteolysis experiments**

For a comparison of the low mol. wt. polypeptides of Berne virus, *S. aureus* V8 protease digests were prepared of the 22K, 20K, 19K and 17K proteins which were then separated electrophoretically in a 20% polyacrylamide gel. Fig. 4 shows that only the 22K polypeptide remained unaffected by the enzyme; the small amount of 17K polypeptide found in this lane is probably due to contamination of the 22K material with 20K protein during excision from the gel. In all lanes, undigested material of the original size can be detected. The 20K, 19K and 17K proteins share two small peptides of about 13K and 10K but also show unique hydrolysis products. For the major fragments of the 20K, 19K and 17K proteins, mol. wt. of 17K, 16K and 14K, respectively, were calculated; minor fragments of 19K, 18K and 16K were also noted. The same patterns were observed after electrophoresis in a 17.5% gel (results not shown); boiling of the preparations did not affect the results. The undigested viral proteins and a low mol. wt. marker set were used for the calculations; polypeptides of smaller than about 9K were not resolved under the experimental conditions used. It was concluded that the two smallest polypeptides share sequences with the 20K protein which, in turn, is different from the 22K species.
Identification of RNA-binding protein

Cytoplasmic extracts from infected and mock-infected cells labelled in the presence of [\(^{35}\)S]methionine were incubated with the rabbit immune serum showing preferential reactivity with the 20K protein (Fig. 6, lanes 11 and 12). After PAGE of the precipitates, protein renaturation and blot transfer to nitrocellulose, the filters were treated with PPO/toluene and fluorographed. Fig. 5 (lanes 3 and 4) shows that viral and cellular proteins were efficiently transferred, also in the relevant low mol. wt. region.

For the identification of RNA-binding protein(s) an experiment was performed in parallel using fresh unlabelled lysates (the radioactive preparations had been stored frozen). Before immune precipitation, the preparations were clarified by low-speed centrifugation to remove nuclear material and incubated with \(^{32}\)P-labelled RNA from Berne virus-infected cells as described. Fluorography revealed a single band occurring only in the infected cell extract (Fig. 5, compare lanes 1 and 2) which appeared in a position identical to the 20K polypeptide of the [\(^{35}\)S]methionine-labelled preparation.

It should be noted that the 37K phosphoprotein did not bind RNA to an extent detectable with this technique.

Identification of viral proteins by RIP

Using lysates of [\(^{35}\)S]methionine-labelled Berne virus-infected cells, RIPs were performed with field sera from cattle and horses, as well as with two rabbit hyperimmune sera. As
Fig. 6. Polypeptides in [$^{35}$S]methionine-labelled extracts of Berne virus-infected (odd-numbered lanes) and mock-infected (even-numbered lanes) EMS cells electrophoresed either directly (lanes 5 and 6) or after immune precipitation. Two field sera from cattle with antibody against Lyon-4 virus (lanes 1 to 4), two rabbit anti-Berne virus hyperimmune sera (lanes 7, 8 and 11, 12) and a horse field serum with high neutralizing activity (lanes 9 and 10) were examined by RIP. *S. aureus* cells were used for precipitation of the immune complexes formed with cattle sera, anti-species IgG sera in the remaining reactions. The rabbit serum in lanes 11 and 12 which preferentially recognized the 20K polypeptide was used in the RNA blotting experiments shown in Fig. 5.

Presented in Fig. 6, lanes 1 to 4, proteins with mol. wt. of 22K, 20K, 19K and 17K were recognized by the bovine sera which possessed neutralizing antibodies against Lyon-4 virus (A. Moussa, personal communication; Weiss et al., 1983). No corresponding bands appeared in the precipitates when mock-infected cell lysates were used. It should be noted that the two sera recognized the 19K and 17K fragments of the 20K polypeptide to varying degrees. With the other sera, and the use of a second antibody (instead of Protein A) additional viral proteins of greater than 200K and in the 80K to 120K range were revealed (Fig. 6, lanes 7 and 9). One rabbit antiserum which had been obtained by immunization with purified Berne virus recognized all six proteins (compare lanes 7 and 8), whereas another serum contained antibodies directed mainly against the 20K polypeptide (compare lanes 11 and 12); this preparation was also used for immunoprecipitation in the RNA-binding experiments described above. In contrast, the horse serum which had been selected from field samples for its high neutralizing activity showed pronounced precipitation of the greater than 200K and the 80K to 120K species while giving virtually no reaction with the major low mol. wt. proteins.

**Morphology of the capsid**

Attempts to visualize the core structure of Berne virus in gradient fractions of detergent-treated material were unsuccessful due to the low particle concentration. Consequently, we have treated preparations of semi-purified virus directly on the electron microscope grid. Most convincing results were obtained using diethyl ether. In Fig. 7 the internal virion structure appears as an elongated particle with rounded ends measuring about 150 nm in length and 27 to 36 nm in diameter; uranyl acetate staining disclosed a transverse striation with a periodicity of about 4 nm.
Fig. 7. Electron micrograph of the nucleocapsid of Berne virus released by treatment with ethyl ether. Negative staining with uranyl acetate; bar marker represents 100 nm.

DISCUSSION

The 20K polypeptide is the most prevalent in the Berne virion, accounting for about 84% of the protein mass (Fig. 2). It was detected in gradient fractions with maximum infectivity (1.16 g/ml, Fig. 1), but also in the intermediate peak (ρ = 1.11 g/ml) which contains about 10% of the infectivity of the virion peak (Weiss et al., 1983). The 20K protein is part of a substructure which was liberated from the virion by treatment with Triton X-100 and which subsequently could be pelleted through sucrose of virion density (Fig. 2). In infected cells, it was present in a component of nucleoprotein density (1.36 g/ml in CsCl; Fig. 3) which contained hot TCA-soluble phosphate label indicative of nucleic acid (M. C. Horzinek & J. Ederveen, unpublished observations). In blotting experiments, the 20K protein was the only RNA-binding polypeptide species detectable in the infected cell lysate (Fig. 5); its isoelectric point is in the neutral to basic range, as indicated by two-dimensional PAGE (M. C. Horzinek & J. Ederveen, unpublished observations). These properties qualify the 20K species as the main protein constituent of the nucleocapsid. Nucleocapsid proteins of most enveloped RNA viruses are in the 30K to 60K mol. wt. range; exceptions with low mol. wt. are the Flaviviridae, equine arteritis virus and three genera of the Bunyaviridae family (Matthews, 1982). The 20K protein of Berne virus is phosphorylated as are the nucleocapsid proteins of, for example, corona- and rhabdoviruses.

Second in abundance (about 13% of the virion protein mass) was the 22K protein which occurred in a non-infectious structure of low density (1.07 g/ml) present in media from infected cultures; treatment with Triton X-100 resulted in its quantitative conversion into slowly sedimenting material, an observation indicative of its membrane nature. When virions were submitted to the same treatment, material with a similar sedimentation behaviour was generated. The 22K polypeptide is probably glycosylated since it is adsorbed to lectins (M. C. Horzinek & J. Ederveen, unpublished observations). These observations support the conclusion that the 22K protein is part of the envelope of Berne virus. Another envelope constituent, probably the peplomer, may be assumed in the 80K to 120K range as indicated by the exclusive RIP obtained with a highly neutralizing field serum from a horse (Fig. 6, lanes 9 and 10). Finally, the phosphorylated polypeptide of 37K is probably also associated with the viral envelope since it is present both in the low density (1.07 g/ml) and the virion peak (Fig. 1); it is devoid of RNA-binding properties. A phosphorylated envelope glycoprotein has been identified in Kern Canyon virus (Sokol & Clark, 1973), a rhabdovirus.
In limited proteolysis experiments using an enzyme which specifically cleaves peptide bonds on the COOH-terminal side of glutamic acid (Houmard & Drapeau, 1972), the 22K species remained unaffected. In contrast, the 20K, 19K and 17K polypeptides were found to possess two common low mol. wt. fragments of about 13K and 10K, and additional cleavage products appearing consistently 3K and 1K smaller than their uncleaved parent molecules. The 17K polypeptide which co-sedimented with the high density nucleocapsid structure is thought to represent a defined proteolytic cleavage product; the same is probably true for the 19K species as suggested by the Cleveland patterns (Fig. 4); in Fig. 8 a schematic interpretation of the results is given.

In RIP experiments, the 20K polypeptide was recognized preferentially by heterologous (cattle) field sera; it also induced a good response in the rabbit. In contrast, a pronounced signal only in the 80K to 120K region was obtained with a highly neutralizing field serum from a horse, indicating surface determinants (peplomer proteins; Fig. 6). The 20K protein was also discovered in a RIP control preparation which contained a (purchased) goat anti-rabbit IgG serum alone (J. Ederveen & M. C. Horzinek, unpublished results); this chance observation is explained by our recent finding that antibodies to Berne-related viruses are common in goats and other ungulates (Weiss et al., 1984). Internal proteins are likely candidates for carrying evolutionarily conserved, broadly cross-reactive antigenic determinants since they are not exposed to selective pressure by antibodies in populations of different hosts.

REFERENCES


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