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The Peplomers of Berne Virus

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SUMMARY

Using [^{3}H]glucosamine and [^{3}H]mannose labels, two virus-specific glycosylated polypeptide species with M_r values of about 200 000 (200K) and in the 75K to 100K range, respectively, were recognized in Berne virus-infected embryonic mule skin cells. In purified virions only the latter glycoprotein occurred. Concanavalin A was bound to the virion as evidenced by reduction in infectivity. Analyses using SDS–PAGE, blotting and glycoprotein identification with concanavalin A and horseradish peroxidase showed coincidence of the virion glycoprotein signals with the maximum infectivity and haemagglutinating activity in an isokinetic sucrose gradient. Polyclonal rabbit immune serum and a neutralizing and haemagglutination-inhibiting monoclonal antibody raised against Berne virus recognized both the 75K to 100K and the '200K' glycoproteins. Using tunicamycin, a concentration-dependent inhibition of infectivity was noted; however, non-infectious particles containing the two major polypeptides (20K and 22K) were released from the cells in small quantities. The glycoproteins were absent from cytoplasmic extracts and a novel polypeptide of about 150K was identified instead. Translation of poly(A)-selected intracellular RNA from infected cells in a rabbit reticulocyte cell-free system also resulted in the appearance of a new high M_r polypeptide (about 170K). Using pulse–chase labelling and radioimmunoprecipitation, suggestive evidence for a precursor–product relationship between the intracellular '200K' and the virion glycoproteins has been obtained. These experiments identify the *N*-glycosylated proteins in the 75K to 100K range as constituents of the peplomeric envelope projection of Berne virus; they probably arise by post-translational processing of a 150K to 170K precursor molecule involving glycosylation and subsequent cleavage.

INTRODUCTION

The history, purification and partial characterization of Berne virus (BEV), a representative of a newly proposed family of enveloped RNA viruses, have been reviewed recently (Horzinek & Weiss, 1984; Weiss & Horzinek, 1986*c*). BEV measures 120 to 140 nm at its largest diameter and consists of a peplomer-bearing envelope and an elongated core which may be bent into an open torus within the membrane (Weiss *et al.*, 1983). The core is of tubular structure and has a morphology indicative of helical symmetry; it contains a single-stranded RNA of positive polarity (M. C. Horzinek, J. Ederveen, W. Spaan & M. Weiss, unpublished results) and a 20000 M_r (20K) phosphoprotein with RNA-binding properties. This structure consequently is identified as the viral nucleocapsid (Horzinek *et al.*, 1985).

By definition, the remaining proteins must be attributed to the viral envelope. The 22K species (Horzinek *et al.*, 1984), which is second in abundance, has been identified as the main envelope polypeptide; in infectious culture media it is present in a low density substructure which is converted into slowly sedimenting material by treatment with non-ionic detergents. A 37K phosphoprotein is also probably envelope-associated (Horzinek *et al.*, 1985).

In the present paper, experiments are reported which show that BEV possesses only one cluster of antigenically related glycosylated polypeptides which constitute the peplomeric surface projections.

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), and antibiotics. The FCS was pre-tested by neutralization assay for the absence of antibodies against BEV. Confluent EMS cell cultures were rinsed with phosphate-buffered saline (PBS) containing 50 mg/l DEAE-dextran and inoculated with the P138/72 strain of BEV at a multiplicity of about 0·05. Supernatants were harvested at 24 h post-infection, at the beginning of c.p.e. The haemagglutinating activity of BEV preparations was assayed as described by Zanoni *et al.* (1986).

Immune sera and monoclonal antibodies. A polyclonal immune serum obtained after immunization of rabbits with purified BEV and a serum with high neutralizing antibody activity obtained from a naturally infected horse were used in radioimmunoprecipitation (RIP). In addition, a neutralizing monoclonal antibody (MAb) was selected from a panel of hybridomas raised against purified BEV; the properties of the MAbs will be reported elsewhere (B. Kaeffer & M. C. Horzinek, unpublished).

Isotope labelling. For [^{35}S]methionine labelling, the medium was removed at 8 h after infection with virus at a multiplicity of about 1, and 1 ml methionine-free MEM supplemented with 2% FCS and containing 200 μCi of the labelled amino acid (1230 Ci/mmol, Amersham) was used per 35 mm^2 plastic Petri dish (Costar); labelling was allowed to proceed from 8 to 15 h post-infection. For labelling with radioactive sugars MEM supplemented with 2% FCS and containing 1/10 of the standard glucose concentration was used and D-[6-^{3}H]glucosamine hydrochloride (34·6 Ci/mmol, Amersham) and D-[2-^{3}H(N)]mannose (27·2 Ci/mmol, New England Nuclear) were added to final concentrations of 100 μCi/ml. After the labelling period the supernatants were clarified by low-speed centrifugation and pelleted at 100000 ***g*** for 1 h; the pellets were resuspended in 100 μl volumes of TES buffer (0·02 M-Tris–HCl pH 7·4, 1 mM-sodium EDTA, 0·1 M-NaCl) and analysed directly or stored at −20°C. The monolayers were rinsed once with TES buffer and lysed with 0·5% Triton X-100 and 0·5% 1,5-naphthalenedisulphonic acid disodium salt (Eastman Kodak) in 200 μl TES (lysis buffer). After centrifugation for 5 min at 10000 ***g***, the supernatants were analysed directly or after immune precipitation.

The methods for RIP and fluorography have been described previously (Horzinek *et al.*, 1982, 1985). When monoclonal antibodies were used, 2 μl of a rabbit anti-mouse IgG serum (Miles Yeda, Rehovot, Israel) was added to the immune complexes (200 μl hybridoma supernatant, 40 μl cell lysate, overnight incubation at 4 °C) before precipitation with 60 μl 3 M-KCl and 50 μl of a 10% suspension of *Staphylococcus aureus* cells.

Pulse–chase labelling. Confluent EMS cell monolayers in 35 mm plastic Petri dishes were infected with BEV at a m.o.i. of 0·4 or mock-infected. The medium was removed 15 min before labelling and 0·5 ml methionine-free MEM was added; a 15 min pulse with 100 μCi [^{35}S]methionine was performed at 12 h post-infection followed by different periods of chase with DMEM/2% FCS containing five times the normal amount of unlabelled methionine.

Influence of concanavalin A (Con A) on BEV infectivity. Preparations of BEV were passed through a PD-10 column (Sephadex G-25, Pharmacia) equilibrated with PBSK (PBS containing 0·1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$) and adjusted to a final concentration of 5 μg/ml Con A (Sigma). Control preparations contained 50 mM-methyl α-D-mannopyranoside (Sigma), a specific substrate of this lectin. After incubation at 0 °C for 1 h titrations were made in PBSK and the infectivity was determined in EMS cells.

Identification of Con A-binding proteins. This blotting procedure for the identification of unlabelled glycoproteins was essentially as described by Glass *et al.* (1981) and Clegg (1982). Fractions from an isokinetic gradient (Spinco SW27 rotor run at 3166 × 10^7 rad^2/s at 4 °C, top sucrose concentration 10%, assumed particle density 1·2 g/ml; van der Zeijst & Bloemers, 1976) were analysed by SDS–PAGE and transferred to nitrocellulose filters (BA-85, Schleicher & Schüll) in an LKB electroblotting device. The filters with the transferred proteins (overnight blotting at 0·2 A and 4 °C in 25 mM-Tris, 192 mM-glycine and 20% methanol) were subsequently incubated for 1 h at room temperature on a rocking platform with blocking buffer (bovine serum albumin, Sigma fraction V, 3 g/100 ml in PBS; the protein had been treated with periodic acid to remove lectin-binding glycoproteins). Subsequently, the filters were incubated with 10 μg/ml Con A in PBSK containing 0·5% Triton X-100 for 60 min at room temperature. After extensive washings with PBSK/Triton X-100, the blots were incubated with horseradish peroxidase (50 μg/ml, Boehringer) for 1 h at room temperature and, after a final wash, with the substrate solution (3,3-diaminobenzidine.4 HCl, Serva, Heidelberg, F.R.G.; 0·5 mg/ml in PBS and 0·075% H_2O_2). The reaction was stopped by washing the filters in tap water and drying.

Tunicamycin (TM) experiments. TM (Sigma) was stored at −70 °C as a stock solution containing 50 μg/ml and used at a concentration of 0·5 μg/ml in DMEM supplemented with 2% FCS. At 5 h post-infection, infected and

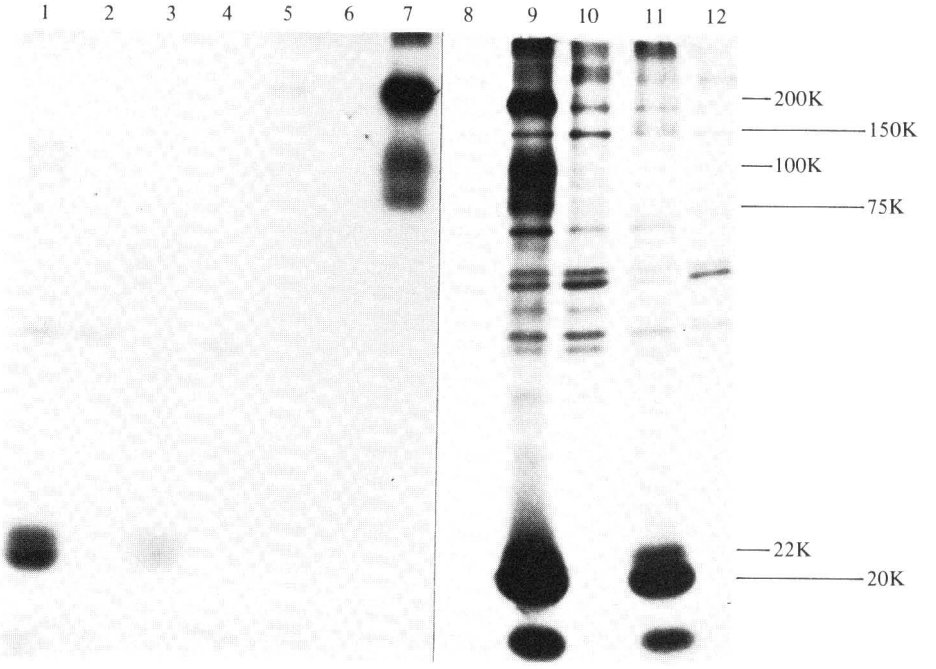

Fig. 1. SDS–PAGE analysis of pellet material after ultracentrifugation of supernatants from EMS cells infected with BEV (lanes 1 and 3) or mock-infected (lanes 2 and 4) and labelled with [^{35}S]methionine in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of TM (0·5 μg/ml). The remaining lanes show analyses of lysates from infected (odd-numbered lanes) and mock-infected EMS cells (even-numbered lanes) after immune precipitation using a polyclonal rabbit immune serum. [^{3}H]Glucosamine- (lanes 5 and 6) and [^{3}H]mannose- (lanes 7 and 8) labelled preparations have been analysed as well as [^{35}S]methionine material labelled in the presence (lanes 11 and 12) or absence (lanes 9 and 10) of TM. Note 150K signal in lane 11.

mock-infected EMS cells were overlaid with this medium and harvested at 15 h post-infection. To study the influence of TM on viral growth, the drug was added at different times following infection and the culture media were harvested at 25 h post-infection.

Translation of mRNA in vitro. RNA was extracted from virus-infected and mock-infected cellular lysates. An equal volume of TES buffer containing 2% SDS and 7 M-urea was added, followed by the same volume of redistilled phenol and chloroform in equal proportions, saturated with TES buffer containing 0·1% 8-hydroxyquinoline. To the pooled aqueous phases of three subsequent extractions 2 vol. ethanol (96%) and sodium acetate pH 5·5 (final concentration 0·1 M) were added for precipitation. A rabbit reticulocyte system was prepared and treated with micrococcal nuclease as described by Jackson & Hunt (1983). For translation, 5 μl of the lysate was mixed with 0·5 μl [^{35}S]methionine (sp. act. 1230 Ci/mmol) and 0·5 μl of the RNA preparation (about 1 μg). After incubation for 1 h at 30 °C samples were treated with Laemmli's sample buffer and analysed by PAGE.

RESULTS

Sugar labelling

For the purpose of identification of viral glycoproteins, carbohydrate labelling was performed and cell lysates were analysed. As demonstrated in Fig. 1, radioactive mannose and to a far lesser extent glucosamine were incorporated into both a high M_r polypeptide (approximately 200K) and into heterogeneous material in the 75K to 100K range (lanes 7 and 5, respectively) which were recognized by a polyclonal rabbit immune serum. Corresponding bands were absent in the mock-infected preparations (lanes 6 and 8). The [^{35}S]methionine-labelled samples analysed in parallel (lanes 9 and 10) showed virus-specific proteins of the same size and in addition the nucleocapsid (20K) and envelope proteins (22K). It should be noted that no sugar label was

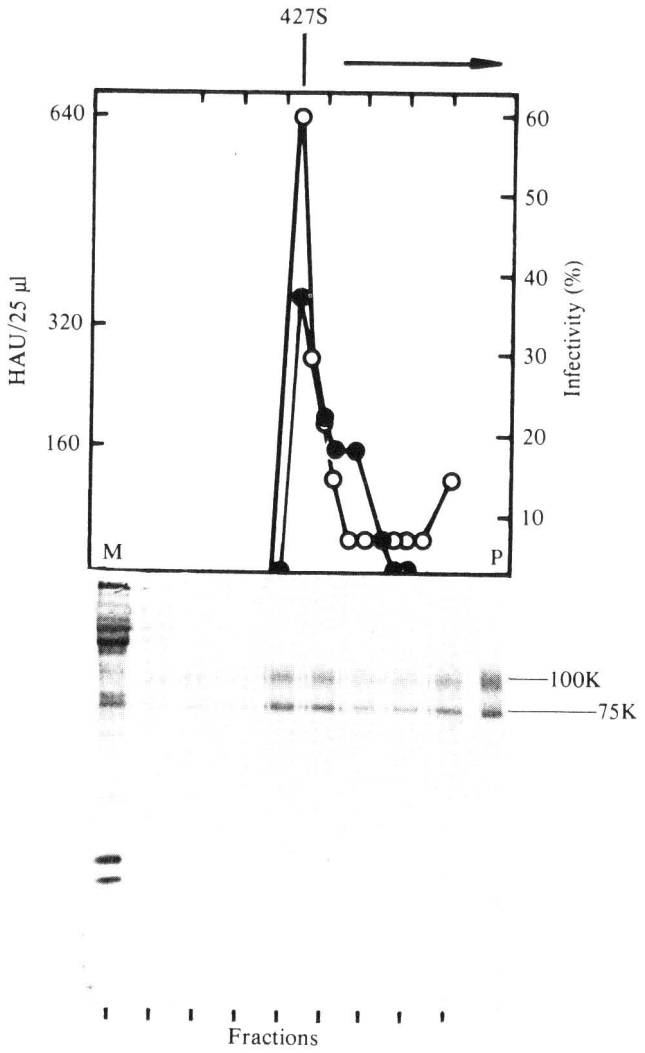

Fig. 2

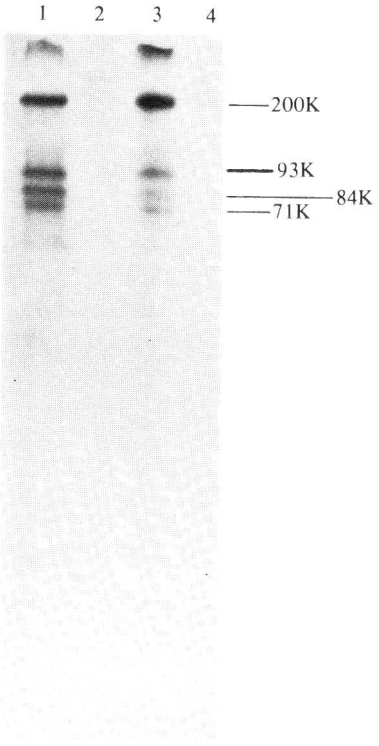

Fig. 3

Fig. 2. ConA/peroxidase recognition of BEV glycoproteins in fractions from an isokinetic sucrose gradient analysed by PAGE and blotted onto nitrocellulose. Sedimentation was from left to right. Distribution of infectivity (ID_{50}; ●) and haemagglutinating activity (HAU; ○) is shown in the graph. The last lane on the right shows the analysis of pelleted BEV (P); note that most Con A-binding material remains at the meniscus (M).

Fig. 3. SDS–PAGE analysis of immunoprecipitates using lysates of BEV-infected (odd-numbered lanes) and mock-infected EMS cells (even-numbered lanes) labelled in the presence of [^{3}H]mannose; a polyclonal rabbit immune serum (lanes 1 and 2) and the BEV-neutralizing monoclonal antibody 11A1D2H1 (lanes 3 and 4; B. Kaeffer, unpublished results) were used. In contrast to [^{35}S]methionine-labelled preparations, discrete bands are seen in the 75K to 100K M_r range.

present in the 22K polypeptide. Incorporation of label into extracellular virions was not sufficient for analysis.

Lectin recognition

The presence of accessible sugar residues on the virion surface was indicated by the results of Con A experiments. BEV aggregation by the lectin was apparent from the reduction of titres by

99·5 to 99·7%, when the infectivity titrations were read 3 days after infection; later readings (5 days post-infection) resulted in lower values (98·2%) which may be expected when single cells are infected near the dilution endpoint by virus aggregates and their early cytopathology escapes detection. Pretreatment of the cells with Con A did not significantly influence infectivity titres. Since labelled glycoproteins could not be demonstrated in extracellular virus due to low levels of radioactive sugar incorporation the Con A peroxidase blotting procedure was employed. As shown in Fig. 2, Con A-binding proteins in the 70K to 100K range were present in the density gradient at the expected 400S position of highest infectivity and haemagglutinating activity. The '200K' glycoprotein was not encountered in the virion, in confirmation of earlier observations (Horzinek *et al.*, 1984).

Immune precipitation

In order to identify the virion substructure responsible for induction of neutralizing antibodies, RIPs were performed. For this purpose the neutralizing and haemagglutination-inhibiting MAb 11A1D2H1 was selected from a panel of hybridomas produced in the Utrecht laboratory using splenocytes of BEV-immunized BALB/c mice (B. Kaeffer *et al.*, unpublished results); a neutralizing polyclonal serum obtained after immunization of rabbits with purified BEV was included. When [^{35}S]methionine-labelled cellular extracts were used as antigen, a diffuse band in the 75K to 100K region of the gel was detected with both antibody preparations (results not shown), similar to the patterns obtained before with polyclonal sera (Horzinek *et al.*, 1984). In order to identify discrete molecular species, [^{3}H]mannose label was used. It can be seen in Fig. 3 that the polyclonal as well as the monoclonal antibody preparations recognized a '200K' polypeptide and additional discrete proteins with M_r values of 93K, 84K and 71K in lysates from BEV-infected cells. A faint 65K glycoprotein band and another 50K polypeptide (not shown) have been observed, especially in analyses where the higher M_r structures were weak or absent. Using the *t*-test at a 95% probability level, M_r values of between 102 and 91 (n = 9), 88 and 84 (n = 9), and 76 and 73 (n = 12) were calculated for these polypeptides; we will refer to them collectively as P proteins.

Identification of a precursor

The presence of two classes of glycosylated viral polypeptides in infected cells (of which only one becomes part of the mature virion) suggests that the larger structure might serve as a precursor for the smaller ones. Consequently, we performed pulse–chase experiments where after a 15 min labelling period excess unlabelled methionine was added to the cultures for different periods of time; the resulting products were analysed by SDS–PAGE after immune precipitation using a polyclonal serum. As shown in Fig. 4, there was a gradual increase in the P protein label with a concomitant fading of the signal in the '200K' region.

Tunicamycin inhibition

We then tested the influence of TM on BEV infectivity. A concentration-dependent effect on extracellular infectious virus was noted in the 0·03 to 1 μg/ml range. At higher concentrations of the antibiotic a toxic effect on the EMS cells was noted. At a concentration of 0·1 μg/ml in the medium, a 99% reduction of infectivity was recorded. The inhibitory effect was pronounced only during the first 8 h of infection when titrating the 25 h yield of BEV. When the drug was added 9 h post-infection or later the yield was unaffected.

The following experiments, performed in the presence of 0·5 μg/ml TM were intended to study its effect on the individual viral proteins. First, it was demonstrated that sedimentable particles were released from cells in the presence of the drug, although only in small amounts. In ultracentrifuge pellets from the supernatants of TM-treated cultures labelled with [^{35}S]methionine both the 22K and 20K proteins were detected whereas material from mock-infected cultures showed no signal in this range (results not shown). Also RIP (Fig. 1, lanes 1 to 4) resulted in the

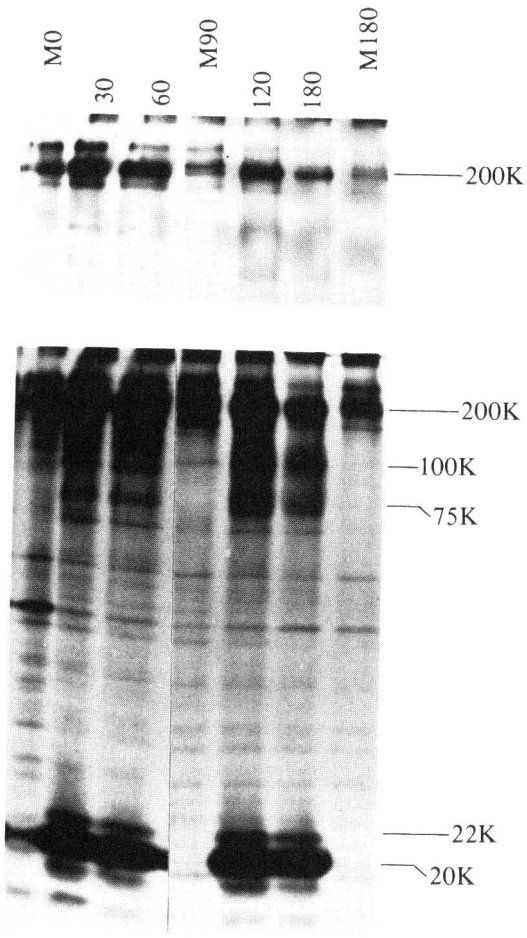

Fig. 4

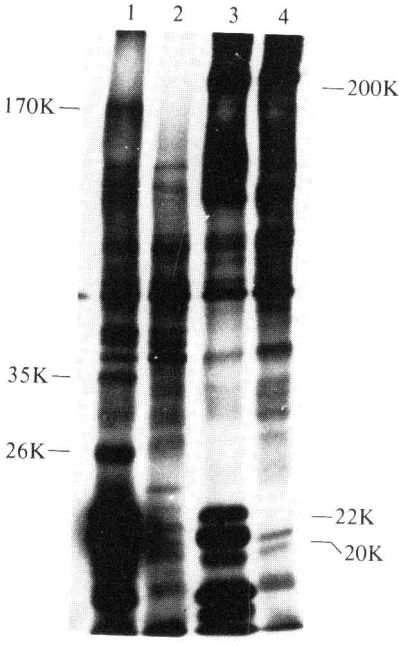

Fig. 5

Fig. 4. Pulse–chase experiment showing the decrease in intensity of the '200K' glycoprotein label (upper part, short exposure) accompanied by an increase of the signal in the 75K to 100K region with longer pulses (lower part, long exposure). Above the lanes the time (min) of chase is indicated; the lanes labelled M show the effect of chase on mock-infected cultures for the indicated periods of time.

Fig. 5. *In vitro* translation products of total intracellular RNA extracted from infected (lane 1) and mock-infected (lane 2) EMS cells. In the two other lanes, [^{35}S]methionine-labelled proteins in extracts from infected (lane 3) and mock-infected (lane 4) cells have been electrophoresed for comparison. M_r values of virus-specific products from the *in vitro* system (left) and from infected cells (right) are given.

demonstration of both low M_r virion polypeptides. No signal was obtained from the P proteins, but their absence cannot be proven owing to their low abundance in the virion. When lysates of infected cells were examined after RIP neither '200K' protein nor P proteins were detectable (Fig. 1, lanes 11 and 12). Instead, new polypeptide species appeared in the TM lane which were absent in the mock-infected sample. The M_r of the more prominent protein was estimated as 150K and another 115K species was seen. The control lanes (1 and 2) in this experiment confirmed that the '200K' glycoprotein was not part of the virion but remains in the cell.

Translation in vitro

On the assumption that unglycosylated intracellular precursors of the '200K' species should be detectable in a cell-free *in vitro* system, we programmed rabbit reticulocyte lysates with total

RNA extracted from BEV-infected or mock-infected EMS cells using cellular extracts labelled *in vivo* as a reference. As depicted in Fig. 5, a high M_r structure which was distinctly smaller than the glycosylated '200K' *in vivo* product was synthesized. No signal was seen in the 170K region in the lysate programmed with RNA from mock-infected cells. Additional unique polypeptides of 35K (probably the unphosphorylated form of the 37K matrix protein), 26K (of unknown specificity), the 22K envelope protein and the 20K nucleocapsid protein (and its proteolytic cleavage products: Horzinek *et al.*, 1985) were visible.

DISCUSSION

In our attempts to define further the structural characteristics of the Toroviridae, a proposed new family of enveloped animal RNA viruses (Horzinek & Weiss, 1984), we here present evidence on the identification of the glycoproteins of BEV, the designated prototype. We conclude that glycoproteins in the 75K to 100K range (referred to as 120K to 80K in all earlier studies from the Utrecht laboratory) represent the building blocks of the viral peplomer, the most peripheral structure of the particle. This concept is supported by the observation that a neutralizing and haemagglutination-inhibiting MAb recognized these glycoproteins and that the sugar residues are accessible to Con A as shown by the aggregation experiments. Neutralizing MAbs have been shown to recognize virion surface projections e.g. in coronaviruses (Koolen *et al.*, 1984; Wege *et al.*, 1984; Talbot *et al.*, 1984), as has induction of neutralizing antibody by immunization with isolated peplomers (Garwes *et al.*, 1979; Wege *et al.*, 1984; Talbot *et al.*, 1984; Mockett *et al.*, 1984). The significance of the different M_r species, and their polymerization to form the peplomer, remain to be elucidated.

The pulse–chase experiments, in which we identified the '200K' glycoprotein as the precursor of the P polypeptides, support the notion that the peplomer is constructed from post-translationally processed glycopolypeptides. The presence of a novel 150K protein in tunicamycin-treated cells and of a 170K protein in translation lysates *in vitro* indicates the size of the unglycosylated primary translation product. The numerical variations reflect the imprecision of the M_r determinations in the high molecular weight range rather than true differences. The observation that the MAb directed against an epitope on the P glycoprotein (see Fig. 3) also recognized the '200K' structure further supports our conclusion that it represents an intracellular precursor of the smaller polydisperse glycoprotein species. This antigenic relationship is further supported by earlier RIP experiments where a rabbit serum raised against purified BEV recognized both proteins in lysates of infected cells (Horzinek *et al.*, 1985: see Fig. 6, lanes 7 and 8). The heterogeneity of the structure is reminiscent of the matrix protein E1 in coronaviruses (Rottier *et al.*, 1981); however, we do not know whether it is due to different degrees of glycosylation or to variations in polypeptide chain length.

The present study supports our recent conclusions drawn from observations with Breda virus (Woode *et al.*, 1982), another torovirus. Using surface radioiodination of virus particles purified from faeces of calves with diarrhoea, major polypeptides of 85K and 105K were identified and an additional 65K species was visible. Neutralizing sera obtained after immunization of mice with one serotype recognized these proteins in preparations of another serotype of Breda virus (Koopmans *et al.*, 1986).

In Fig. 6, a schematic representation of torovirion anatomy is given and a nomenclature for the proteins is proposed. The peplomeric surface projections (P) are constructed from glycoprotein molecules of 100K and smaller which possess (at least) one common epitope. Western blotting experiments are presently being performed to exclude possible co-precipitation of aggregated polypeptide chains. Further studies are needed to localize specifically the 37K matrix phosphoprotein M; it can be radioiodinated together with the major envelope protein E in intact virions where the N (nucleocapsid) protein remains unlabelled (Weiss & Horzinek, 1986*c*) and therefore is probably exposed. Either or both the E and M polypeptides may have a role in nucleocapsid recognition during budding (Weiss & Horzinek, 1986*a*). The membrane proteins may effectively shield the lipid bilayer, thereby contributing to the extreme stability of BEV to deoxycholate (Weiss & Horzinek, 1986*b*).

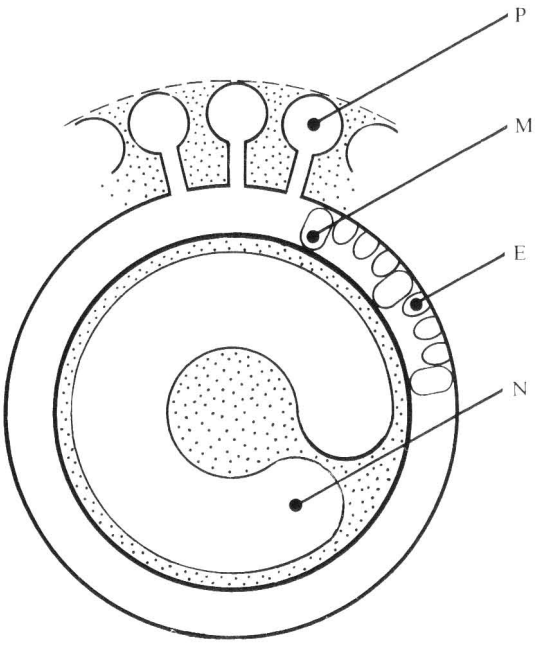

Fig. 6. Schematic representation of the architecture of Berne virus, the proposed prototype of the new family Toroviridae. The localizations of the peplomer glycoprotein (P, M_r 75K to 100K), matrix phosphoprotein (M, M_r 37K), envelope protein (E, M_r 22K) and nucleocapsid phosphoprotein (N, M_r 20K) are indicated.

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