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# Primary Structure<sup>1</sup> and Post-translational Processing of the Berne Virus Peplomer Protein

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The nucleotide sequence of the peplomer (P) protein gene of Berne virus (BEV), the torovirus prototype, was determined. The gene encodes an apoprotein of 1581 amino acids with an  $M_r$  of about 178K. The open reading frame was cloned behind the T7 RNA polymerase promoter and its translation product was identified as the BEV P protein precursor by *in vivo* expression and immunoprecipitation. The deduced amino acid sequence contains a number of domains which are typical for type I membrane glycoproteins: an N-terminal signal sequence, a putative C-terminal transmembrane anchor, and a cytoplasmic tail. Eighteen potential *N*-glycosylation sites, two heptad repeat domains, and a possible "trypsin-like" cleavage site were identified. The mature P protein consists of two subunits and their electrophoretic mobility upon endoglycosidase F treatment strongly suggests that the predicted cleavage site is functional *in vivo*. The heptad repeat domains are probably involved in the generation of an intra-chain coiled-coil secondary structure; similar inter-chain interactions can play a role in P protein oligomerization. Using a sucrose gradient assay the P protein was indeed shown to form dimers. The intra- and inter-chain coiled-coil interactions may stabilize the elongated BEV peplomers. @ 1990 Academic Press, Inc.

### INTRODUCTION

Berne virus (BEV) is the prototype and best-studied member of a recently described group of positivestranded animal RNA viruses, which has been proposed to form a new virus family (the Toroviridae; Horzinek and Weiss, 1984; Horzinek et al., 1987). Torovirions are characterized by the unique tubular morphology of their nucleocapsid and by the fact that their envelope is studded with drumstick-shaped projections, resembling the spikes of coronaviruses (Weiss et al., 1983). For BEV (diameter of the disc-shaped virion 120-140 nm) these peplomers measure 20 nm in length (Weiss et al., 1983). Electrophoretic analysis of virion proteins has revealed the presence of four structural polypeptides in BEV particles (Horzinek et al., 1984). A heterogeneous band of  $M_r$  75K-100K is thought to represent the peplomer (P) protein, since it is membrane-associated and is recognized by both neutralizing and hemagglutination-inhibiting monoclonal antibodies (Horzinek et al., 1986; Kaeffer et al., 1989).

In a previous study (Horzinek et al., 1986) the N-glycosylation of the P protein has been described. Fur-

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thermore, it was shown that in BEV-infected cells an approximately 200K *N*-glycosylated precursor of the P protein is synthesized. In immunoprecipitations this product is recognized by monoclonal antibodies directed against the P protein. Pulse–chase experiments supported the idea that the BEV peplomers consist of post-translationally processed glycopolypeptides.

In BEV-infected cells a 3'-coterminal nested set of five viral mRNAs (approximately 25, 7, 2.1, 1.4, and 0.8 kb in length) is synthesized (Snijder *et al.*, 1990a). Except for the smallest one, these RNAs are polycistronic, but only the most 5'-situated open reading frame (ORF) of each mRNA is translated. On the basis of *in vitro* translation studies (Snijder *et al.*, 1988) BEV RNA 2 was proposed to encode the P protein precursor.

Recently, we have cloned the BEV mRNAs (Snijder et al., 1990a). Nucleotide sequence analysis has revealed the presence of an ORF of 4743 nucleotides (nt) in the unique 5' region of RNA 2. In the present paper, we demonstrate that this ORF encodes the precursor of the BEV P protein; in addition to its primary structure, an analysis of possible functional domains and data on the maturation of the P protein are presented.

## MATERIALS AND METHODS

## cDNA synthesis and cloning

The preparation and cloning of cDNA from poly(A)selected RNA from BEV-infected cells has been de-

<sup>&</sup>lt;sup>1</sup> Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. X52506.

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scribed recently (Snijder *et al.*, 1990a). Clones were mapped using colony hybridization techniques and restriction enzyme analysis.

#### Nucleotide sequence analysis

Sequence analysis was performed as described previously (Snijder *et al.*, 1990a). Sequence data were assembled using the computer programs created by Staden (1986). Every nucleotide in the sequence was determined from at least two independent cDNA clones.

#### Computer analysis of the protein sequence

The deduced P protein sequence was analyzed using a hydrophobicity plot (HYDROPLOT) program based on the method of Kyte and Doolittle (1982) and a signal sequence search program (SIGSEQ) based on the method of Von Heijne (1986). Amino acid sequence similarity searches were performed using the FASTP program (Lipman and Pearson, 1985) and the entries in the NBRF data base (release 20.0). More detailed comparisons were carried out by means of the DIA-GON comparison program of Staden (1982).

# Reconstruction and expression of the BEV P protein gene

The BEV P protein gene was reconstructed from cDNA clones 118, 113, 115, and 106 (Fig. 1) by ligation of the following restriction fragments (numbers indicating nucleotide positions in Fig. 2): clone 118 Dral-Hpal (2-1200); clone 113 Hpal-BamHI (1200-1718); clone 115 BamHI-EcoRI (1718-3267); clone 106 EcoRI-Ball (3267-4853). The reconstructed gene was cloned into the Smal site of pBSXho, a pBS (Stratagene) derivative which contains an Xhol linker (5' CCTCGAGG 3') in the filled EcoRI site of the multiple cloning region. The Sall site of pBSXho was digested and filled, and the P protein gene was isolated from pBSXho by digestion of the Xhol site. The gene was recloned between the Xhol and Smal sites of plasmid pTUG31, a pAR2529 (Fuerst et al., 1986) derivative which contains an extended multiple cloning region (H. Vennema et al., manuscript in preparation). The resulting construct, pTP, contains the complete BEV P protein gene downstream of a T7 promoter.

Expression of the recombinant P protein gene was studied by transfection (Gorman, 1985) of HeLa cells  $(1 \times 10^6 \text{ cells in a } 10\text{-cm}^2 \text{ dish})$  with 5 µg of pTP DNA. Earlier, the cells had been infected (at an m.o.i. of 5 PFU) with recombinant vaccinia virus vTF7-3, in which the expression of the T7 polymerase gene is under the control of a vaccinia promoter (Fuerst *et al.*, 1986).

#### Metabolic labeling of viral proteins

Embryonic mule skin (EMS) cells were infected with the P138/72 strain of BEV at an m.o.i. of 10 as described previously (Snijder *et al.*, 1988). Labeling experiments were carried out in 10-cm<sup>2</sup> petri dishes. The culture supernatant was removed 15 min before labeling and replaced by methionine-free or cysteine-free medium. A 30-min pulse with 100  $\mu$ Ci/ml [<sup>35</sup>S]methionine or [<sup>35</sup>S]cysteine was performed at 10 hr postinfection (p.i.). The pulse was followed by chase periods of 0, 30, or 150 min in normal DMEM containing 2% fetal calf serum. vTF7-3 pre-infected, pTP-transfected HeLa cell cultures were labeled similarly at 14 hr p.i. (= 13 $\frac{1}{4}$ hr post-transfection). Cells were lysed in 300  $\mu$ l TESV buffer containing 1% Triton X-100 (TX-100) as described by Vennema *et al.*, (1990).

In order to study P protein oligomerization, cells were infected as described above, labeled continuously from 10 to 16 hr p.i. with 100  $\mu$ Ci/ml [<sup>35</sup>S]methionine, and lysed in MNT buffer containing 1% TX-100 as described by Doms *et al.*, (1988).

# Immunoprecipitation and endoglycosidase treatment

Polyclonal immune sera, obtained after immunization of rabbits with purified BEV, and monoclonal antibody 6B10 (Kaeffer *et al.*, 1989) were used to precipitate the BEV P protein. Immunoprecipitations were performed as described by Vennema *et al.*, (1990).

For endoglycosidase assays viral proteins were immunoprecipitated from 100  $\mu$ l of cell lysate. Subsequently, one-third of the dissolved precipitate (10  $\mu$ l) was treated with endoglycosidase H as described by Vennema *et al.*, (1990). The remaining 20  $\mu$ l of the sample was mixed with 40  $\mu$ l endoglycosidase F buffer (50 m*M* EDTA; 1% Nonidet-P40; 1% 2-mercaptoethanol; 0.125% SDS; 100 m*M* sodium citrate, pH 6.5) and divided into two 30- $\mu$ l aliquots. To one of these samples 5 mU endoglycosidase F (endo F; Boehringer-Mannheim) was added. All samples were incubated at 37°C for 16 hr and analyzed by SDS–PAGE and fluorography (Snijder *et al.*, 1988).

#### **Oligomerization assay**

The quaternary structure of the BEV P protein was studied by sucrose gradient centrifugation as described by Doms *et al.*, (1988). A total of 500  $\mu$ l of cell lysate (in MNT buffer containing 1% TX-100; see above) was loaded onto an 11.5 ml linear 5–20% sucrose gradient, which was spun in an SW41 rotor (Beckman) for 12 hr, 38,000 rpm at 4°. Following a two-fold dilution of the gradient fractions in TESV buffer, an



Fig. 1. Restriction map and sequencing strategy of the BEV peplomer protein gene. A map from about 2 to 7 kb upstream of the poly(A) tail is shown. Restriction sites are indicated by vertical bars and one-letter codes: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; N, NdeI; P, PstI; S, NsiI; V, PvuII. The position of clones used in sequence analysis is indicated; arrows indicate the sequencing direction. The P protein ORF and the flanking ORFs of the BEV polymerase (POL) and envelope (E) protein genes are represented by open boxes. The initiation and termination codons of the P protein ORF are indicated by their nucleotide distance to the start of the poly(A) tail.

anti-BEV rabbit serum was used to immunoprecipitate the viral proteins as described above. Using an identical sucrose gradient, the sedimentation behavior of the marker proteins apoferritin (from horse spleen; Pharmacia), catalase (from beef liver; Pharmacia), lactate dehydrogenase (from beef heart; Pharmacia), and albumin (from bovine serum; Pharmacia) was analyzed. Marker proteins were TCA-precipitated and analyzed directly by SDS-PAGE and Coomassie brilliant blue staining.

#### RESULTS

### Nucleotide and amino acid sequence

We have recently described the analysis of cDNA clones covering about 10 kb (starting at the 3' end) of the BEV genome (Snijder *et al.*, 1990a). In this nucleotide sequence a large ORF (4743 nt) was identified at positions 6684 to 1941 upstream of the poly(A) tail. The sizes of BEV RNAs 2 and 3, about 7 and 2 kb, respectively, suggested that this ORF is translated from RNA 2. Northern blot hybridizations confirmed this assumption: probes from the 5' and 3' ends of the ORF hybridized to RNAs 1 and 2, but did not hybridize to RNA 3; a probe from the area upstream of the ORF hybridized to RNA 1 only (Snijder *et al.*, 1990a). The position of the cDNA clones in this region and the sequencing strategy are summarized in Fig. 1.

The nucleotide and deduced amino acid sequence of the ORF and its product are presented in Fig. 2. The ORF is flanked by two short nucleotide sequences (positions 9–16 and 4813–4820) which have been shown to be conserved upstream of each ORF of BEV. They probably represent core promoter sequences involved in the transcription of the BEV subgenomic mRNAs (Snijder *et al.*, 1990a). The AUG-codon at position 61–63 is in a favorable context for use as translation initiation signal (Kozak, 1987). The ORF encodes an apoprotein of 1581 amino acids (aa) with a calculated  $M_r$  of 178,334.

A hydrophobicity plot (Fig. 3) shows that both the amino- and the carboxyl-termini of the protein are hydrophobic. An analysis of the N-terminal region using the method of Von Heijne (1986) predicts that the first 19 aa constitute a signal sequence. The somewhat larger hydrophobic stretch near the C-terminus forms a possible transmembrane anchor. Eighteen potential *N*-glycosylation sites (N-X-S or N-X-T; X may not be P) were identified (Figs. 2 and 3), which could add up to 40K to the  $M_r$  value (178K) of the apoprotein.

Using the Fastp program the amino acid sequence was compared with the entries in the NBRF protein data base, but no significant sequence similarities were observed.

# Reconstruction and expression of the BEV P protein gene

To obtain experimental evidence that this large ORF encodes the BEV P protein, the full-length ORF was reconstructed from cDNA clones 118, 113, 115, and 106 and cloned downstream of the  $\top 7$  promoter, resulting in construct pTP. To express the reconstructed gene in vivo, HeLa cells were infected with recombinant vaccinia virus vTF7-3, which expresses the T7 polymerase, and subsequently transfected with pTP DNA. Metabolic labeling revealed the synthesis of a protein of about 190K which comigrated with the P protein precursor from BEV-infected cells and which was recognized by a monoclonal antibody directed against the BEV P protein. The 190K protein was not detected in nontransfected cell lysates. These data demonstrate that the large ORF encodes the BEV peplomer protein (Fig. 4).

#### Cleavage of the BEV peplomer protein

The identification of a 200K precursor in infected cells and the presence of 75K–100K peplomer-specific proteins in virions (Horzinek *et al.*, 1984, 1986) indicated that the P protein is subject to a post-translational cleavage event. In the amino acid sequence of the BEV P protein a potential "trypsin-like" cleavage site consisting of five consecutive arginine residues was identified at positions 1002–1006. After the removal of a 19 aa signal sequence, cleavage between

M F L C F C A A T V L C F W I N S G A TTTTAAAG <u>TGTTTAGT</u> ACTAGTTTTAGTTATAAGTATAGTGGTGTTAAGTTTGTGCGAAAAA <u>ATG</u> TTTTTATGTTTCTGTGCCGCGACAGTTTTGTGCTTCTGGATTAATTCAGGAGGTGCC .core	0020 0120
D V V P N G T L I F S E P V P Y P F S L D V L R S F S Q H V V L R N K R A V T T	0060
GATGTTGTGCCAAATGGAACATTAATTTTTAGTGAGCCAGTACCTTATCCTTTTTCACTAGATGTTCTTCGTTCG	0240
ISWSYSYQITTSSLSVNSWYVTFTAPLGWNYYTGQSFGTV	0100
ATCTCTIGGTCGTATTCCTACCAAATTACCACCTCATCAATAAGTGTAAATAGTTGGTATGTTACTTTTACTGCACCCCCTAGGGTGGAACTATTACACTGGACAGTCTTTTGGTACTGTG	0360
L N Q N A M M R A S Q S T F T Y D V I S Y V G Q R P N L D C Q V N S L V N G G L	0140
TTAAATGAAAATGAATGATGAGAGAGCCTCTCAGAGTACCTTTACATATGATGTGATGATATCTTATGTAGGTCAACGTCCTAATTTAGATTGTCAAGAACAGTCTTGTTAATGGTGGGTTG	0480
D G W Y S T V R V D N C F N A P C H V G G R P G C S I G L P Y M S N G V C T R V	0180
GATGGTIGGTAGGACTGGTAGAGTIGATAATIGTITTAATGCACCTTGTCATGTIGGAGGAAGACCTGGCTGTTCAATAGGACTACCTTACATGAGCAATGGAGTATGTACAAGAGTT	0600
L S T T Q S P G L Q Y E I Y S G Q Q F A V Y Q I T P Y T Q Y T I T M P S G I L G	0220
TTATCAACAACTCAGTCACCAGGGTTGCAGTATGAAATTTATTCTGGTCAACAATTTGCAGTTTATCAAATTACACCATATACCAATTACCAATGCCATCTGGAATTTAGGT	0720
Y C Q Q T P L Y V D C G T W T P F R V H S Y G C D K V T Q N C K Y T L T S N W V	0260
TATTGTCAACAACCCCATTGTATGTTGATTGTGGTACTTGGACACCATTTAGGGTACATTCTTATGGTTGTGATAAAGTTACTCAGAATTGTAAATAACATTAACATCAATTGGGTT	0840
VAFQNKATAVILPSELIVPVAQKVTRRLGVNTPDYFNLVK	0300
GTIGCGIIICAGAATAAGGCTACTGCTGTTATTTTACCATCTGAGCTAATTGTGCCTGTAGCACAAAAGGTGACTCGGAGGTTAGGTGTTAATACACCAGATTATTTTGGTTGG	0960
Q A Y H Y L S Q A N L S P N Y A L F S A L C N S L Y Q Q S A T L S T L C F G S P	0340
CAGGCTTACCATTATTTGTCACAAGCAAATTTGTCACCTAATTATGCATTGTTTTCGCACTCTGTAATTCACTTTATCAACAATCAGCAACATTGTCAACCTTATGTTTTGGGGTCACCA	1080
FFVAQECYNNALYLPDAVFTTLFSTLFSNDYQINYPLNQV	0380
TTTTTTGTTGCTCAAGAGGTGCTACAACAATGCATTGTATCTGCCTGATGCTGTGTTTACTACTTATTTTCCAACACTTTTTTCTGGGACTATCAAATTAATT	1200
UTAACACAAAATGAGACATTTTTACAATTACCAGCCACAAATTATCAAGGACAAACCCTTTCGCAGGGTAGGATGTTGAATTGTTTAAAGATGCAATAGTTTTTTGGATTTTTTGGATTTTTTGGAT	0420 1320
T K F Y R T N D A P S S D I F V V V A R Q A Q L I R Y G N F R I E Q I N G Y F Q	0460
ACCAAGTTTTACCGGACTAATGATGCTCCCAAGTTCTGATATTTTGTTGTGGGCTCGCCAAGCACAGTTGATCGTTATGGTAATTTTAGAATTGAACAAATAAAT	1440
VKCSSNIISTLEPHPAGVIMIARHHSMWSVAARNSTSFYC	0500
GTTAAGTGTTCATCAACATTTCAACACTTGAACCTCATCCAGCTGGTGTGGTAATGATAGCCAGGCATCATTCAATGTGGTCCGTTGCAGCACGTAATTCTACATCCTTTTATTGT	1560
V T H S L T T F G K L D I S T S W F F H T L A L P S G P V S Q V S M P L L S T A	0540
GTTACACACTCTTTAACAACTTTTGGTAAATTGGATATTTCTACTTCATGGTTTTTTCATACTTTAGCCTTACCAAGTGGCCCGGGTATCCCAAGTGTCTTAGCATTTCAACTGCT	1680
A V G V Y M H P M I E H W I P L L T L A Q S Q Y Q P S F F N I G I N K T I T L T	0580
GCTGTTGGTGTGTATATGCATCCAATGATCGAGCATTGGATCCCCCTTTTAACCTTGGCACAAAGTCAATATCAGCCTAGTTTTTTTAATATTGGTATAAATAA	1800
T Q L Q A Y A Q V Y T A W F L S V I Y V R L P E A R R L T L G V Q L V P F I Q A	0620
ACCCAGTTGCAGGCATATGCTCAAGTTTATACAGCTTGGTTTTTGTCTGTAATTTATGCTTGCGTGCAAGCAA	1920
LLSIKQADLDATDVDSVARYNVLSLMWGRKYAVVNYNQLP	0660
CTTTTGTCAATTAAGCAGGCTGATTTAGATGCAACTGATGTTGTGGATTATAATCGTGGGGTTTCCCTAATGTGGGGGTAGAAAGTATGCTGTTGTGAATTATAATCAATTGCCT	2040
E W T Y P L F K G E I G E S M W F R K K I M P T T E G C Q T S A H F S S I T G Y	0700
GAGTGGACTTACCCGTTATTTAAGGGTGAGATTGGTGAGTCTATGTGGTTTAGAAAGAA	2160
LQFSDYVYIPKYNKVSCPISTLAPSVLQVYEVQSLFVILI	0740
TTACAGTTTTCTGATTATGTTTATATCCCTAAGTATAATAAGGTGTCATGCCCAATACTTTGGCACCATCAGTTTTACAAGTTGAAGTGCAATCCTTGTTTGT	2280
Q C V S G S Y D W Y P G L S G G T A F V Y K S Y K L G T V C V L L P S D V L S T	0780
CAATGTGTTTCAGGCTCTTATGATTGGTACCCAGGGTTATCTGGIGGAACCGCTTITGTGTATAAGTCATATAAGTTGGGTACAGTTYGTGTTCTTTTACCATCTGATGTTCTTTCAACA	2400
G P N I G F Y S G T A L S I V T V Q T T N D V L P N C I G L V Q D N I F T P C H	0820
GGTCCAAATATAGGATTTTATTCTGGAACAGCCTTGTCGATTGCATTGCACTGAAATGAAATGAAGTGTTTTACCTAACTGTATTGGCCTTGTTCAGGATAATATATTTACACCATGTCAT	2520

Fig. 2. Nucleotide sequence and predicted amino acid sequence of the BEV P protein gene. The 5' and 3' flanking sequences of the ORF, containing the postulated core promoters for RNA 2 and 3 synthesis, are also shown. Initiation (>>>) and termination (<<<) codons are indicated. Hydrophobic domains (---), heptad repeats (---), and potential *N*-glycosylation sites (triangled asterisks) are marked. The arrowhead points at the putative "trypsin-like" cleavage site, which is preceded by five arginine residues.

amino acids 1006 and 1007 would generate apoproteins of approximately 111K (987 aa; 7 potential *N*-glycosylation sites) and 65K (575 aa; 11 potential *N*-glycosylation sites). To study the maturation of the BEV P protein precursor a pulse-chase experiment was performed. Labeled viral proteins from infected cell lysates were immunoprecipitated and treated with endoglycosidases F and

PSGCPVRNSYDNYIVCFDSSTYTFKNYHRTTPPVMNVP1Q	0860
CCAAGTGGCTGTCCAGTTCGTAATTCTTATGATAATTATATTGTGTGTTTTGACAGTTCAACTTATACGTTTAAGAATTATCATAGGACAACACCACCTGTTATGAATGTACCAATTCAG	2640
E V P L Q M E I P T V I L Q S Y E L K H T E S V L L Q D I E G G I I V D H N T G	0900
GAAGTACCGTTACAAATGGAAATACCAACAGTAATTTTACAGTCTTATGAGTTAAAACATACTGGATCTGTGCTTTTACAGGACATTGAGGGTGGAATTATTGTAGACCATAATACTGGT	2760
S I W Y P D G Q A Y D V S F Y V S V I I R Y A P P K L E L P S T L A N F T S C L	0940
ICCATTIGGTATCCAGATGGACAAGCCTATGATGTTTCATTTTATGTTTCAGTTATCAGTTATGCACCCCCAAAACTTGAGTTACCATCTTGCACTCTTGCAAATTTTACATCATGTCTT	2880
DYICFGNYQCRTEAQTFCTSMDYFEQVFNKSLISLKTALQ	0980
GATTATATTTGGTTTGGAAATTATCAGTGTCGAACTGAAGCACAGACATTTGCACTTCTATGGATTATTTTGGAGCAAGTTTTTAAAAAGAGGCTTTAAAAAACAGCATTGCAG	3000
D L H Y V L K L V L P E T T L E L T E L T R R R R R R A V Y E F D D T I S L L S E	1020
GATITGCACTATGTTCTTAAGCTTGTATTGCCAGAAACAACATTAGAATTGACTGAATTAACACGTCGTAGAAGACGAGCTGTTTATGAGTTTGATGATACTATATCCCTACTTTCTGAA	3120
S F E R F M S P A S Q A Y M A N M L W W D D A F D G F S L P Q R T G S I L S R S	1060
ICTITIGAGAGATITATGTCACCAGCTICACAAGCCTATATGGCAAATATGTTGTGGTGGGGTGG	3240
P S L S S V S S W N S Y T S R T P L I S N V K T P K T T F N V K L S M P K L P K	1100
CCGTCCCTGTCTCTGTTTCATCTTGGAATICCTATGCTAAGATCTCCTAAGATGTTAAAAGACCCTAAGACGACTTTTAATGTGAAGCTATCTAT	3360
A S T L S K I G S V L S S G L S I A S L G L S I F S I V E D R R V T E L T Q Q Q	1140
GCATCTACTCTTTCAAAGATCG6GTCTGTGTTATCGTCGGGCTTGTCAATTGCTTCTGTTGTAAGATCGACGTGTAACTGAGTTAACAACAACAACAA	3480
I M A L E D Q I T I L T D Y T E K N <u>F K E I Q S S L N T L G Q Q V Q D F S Q V</u>	1180
ATAATGGCTCTTGAAGATCAGATTACTATTTTGACTGATTATACAGAGAAAAATTTTAAAGAGATTCAGTCATCATCAACAACTTGGGACAACAAGTTCAGGATTTTYCACAACAAGTG	3600
T M S L Q Q L S N G L E Q I T Q Q L D K S I Y Y V T A T Q Q Y A T Y M S S L I N	1220
ACTATGTCACTACAGCAACTITCTAATGGTCTTGAGCAGGATTACTCAACAGTTGGATAAGAGTATTTATT	3720
H L T E L A A A V Y K T Q D M Y V T C I H S L Q S G V L S P N C I T P S Q I F Q	1260
CATCITACCGAGCTGGCTGCTGCTGTGTATAAGACGCAAGATATGTATG	3840
LYQVARNLSGQCQPIFSEREVSRFYSLPLVTDANVHNDTY	1300
CTGTATCAAGTTGCTAGAAATTTAAGTGGACAGTGTCAACCAATTTTTTCAGAGCGTGAAGTGTCACGATTTTATTCTCTTCCACTTGTGACTGATGGTGGCATAATGATACATAT	3960
W F S W S I P I T C S N I Q G S V Y K V Q P G Y I V N P T H P T S L Q Y D L P S	1340
TGGTTTTCTTGGTCAATTCCTAACATGTTCTAATATTCAGGGCCTCGTTTATAAGGTGCAGCCAGGGTATATTGTCAATCCAACTCATCCTACCAGCTTACAATATGATTTACCATCT	4080
H V V T S N A G A L R F D D H Y C D R Y N Q V Y L C T K S A F D L Q P S N Y L T	1380
CATGITIGATGACAGAATGCTGGGGGCATTGAGGTTIGATGATCATTATTGTGATAGGTATAATCAGGTCTACCATGTACAAAATCTGCATTTGATCTACAACCGTCAAATTATCTTACA	4200
M L Y S N I S E N V S L T F H P E P R P D P C V Y L S S A L Y C Y Y S D Q C N	1420
ATGCTTTATTCAAATATATCAGAAAATGTTTCATTGACATTTCACCCTGAGCCAAGGACCTGATCCTTGTGTGTACCTTTCTTCATCTGCCCTTTATTGTTATTATTCTGACCAATGTAAT	4320
Q C V V A V G N C S N Q T V T Y R N Y T Y P I M D P Q C R G F D Q I T I S S P I	1460
CAGTGTGTTGTAGCTGTGGGTAACTGCAGTAATCAAACAGTTACATATAGAAATTATAGGTATCCAATTATGGATCCACAGTGTAGAGGTTTTGATCAGAATAACCATTTCTTCGCCTATA	4440
DIGVDFTALPSRPPLPLHLSYVNVTFNVTIPHGLNNTDLV	1500
GATATAGGTGTAGATTITACAGCTTGGCCATCAGGCCACCATTGGCCATCGCCTTGGCATTGGCAATAGGTGCACATTTAAATGGGACAATTGGATTGGGT	4560
U D Y S F K D K I Y E I S K N I T D L H Q Q I L Q V S S W A S G W F Q R I R D F	1540
TIAGATTATAGITITAAAGATAAAATTTATGAGATTTCAAAGAATATAACAGATTTACATCAGCAAATTTGCAGGTTTCTTCTTGGGGCATCTGGTTGGT	4680
LYNLLPTWITWLTLGFSLFSIVISGINIALIFFEWNGKVKK	1580
CTTTATAATCTTCTTCCTACTTGGATTACATGGCTTACTTTAGGTTTTAGTTGTTTGT	4800
S * AGT <u>IAG</u> TCACTI <u>TCTTIAGA</u> AGAAGGTTGCCAAAA <u>TG</u> TTTGAGACCAATTATTGGCCA <<< core >>>	1581 4858

FIG. 2—Continued

H. Endo F cleaves glycans of both the high-mannose and complex type, leaving a single *N*-acetylglucosamine residue attached to the asparagine in the polypeptide chain (Elder and Alexander, 1982). Endo H cleaves only the high-mannose oligosaccharides which are added to the protein in the endoplasmic reticulum (ER; Tarentino and Maley, 1974). In the medial compartment of the Golgi stack these glycans are processed into endo H-resistant "complex" oligosaccharides (Dunphy and Rothman, 1985). Therefore acquisition of Endo H resistance indicates that a glycoprotein has reached the medial cisternae of the Golgi complex.

The results from the pulse-chase experiments revealed that processing of the BEV P protein precursor gave rise to the heterogeneous set of polypeptides in the range of 75K-100K which has been described be-



Fig. 3. Schematic representation of the position of characteristic domains in the BEV peplomer protein. The positions of heptad repeats, hydrophobic segments, potential *N*-glycosylation sites, and the putative cleavage site are indicated. In addition, a hydrophobicity plot of the amino acid sequence is presented. The plot was generated according to the method of Kyte and Doolittle (1982) using a window size of 21. The region above the line is hydrophobic.

fore (Horzinek *et al.*, 1986; Fig. 5). After a 60-min chase, the first cleavage products could be detected (data not shown). In the 150-min chase material two distinct subunits of about 95K and 82K and a minor species of 77K were present. Upon endo F treatment the 82K and 77K proteins both seem to be reduced to a distinct 61K polypeptide species (Fig. 5). The size of the larger P subunit was reduced to about 87K, but in this case endo F treatment did not yield a discrete product. The oligosaccharides linked to the mature P protein were not completely endo H resistant (Fig. 5), but products in the 60K–70K range were not detected in material treated with this enzyme. We therefore assume that at least part of this material had reached the Golgi complex.

The presence of a 61K protein backbone in one of the P protein subunits supports the assumption that







Fig. 5. Cleavage and endo F and endo H treatment of the BEV peplomer protein. Infected EMS cells were pulse labeled for 30 min with [<sup>35</sup>S]cysteine at 10 hr p.i. After 30- and 150-min chase periods, the cells were lysed and viral proteins were immunoprecipitated. Samples were not treated (–) or treated with endo F (F) or endo H (H) and analyzed by SDS-PAGE. The positions of the P protein precursor (calculated at 183K) and its endoglycosidase-treated form (157K) are indicated. After a chase period of 150 min, P protein cleavage products of 95K and 82K were observed. Endo F treatment of this material resulted in the detection of a diffuse 87K band and a distinct 61K polypeptide species.

the stretch of five arginine residues at amino acid positions 1002–1006 functions as a cleavage site during P protein maturation. The considerable differences between predicted and calculated  $M_r$  values for the 111K subunit (and also the P protein precursor) can be explained by deviant migration. Alternatively, further processing of the N-terminal subunit of the P protein could have reduced its  $M_r$ .

#### Quaternary structure of the BEV peplomer protein

Although no significant sequence similarities between the BEV P protein and other proteins were detected (see above), a more detailed comparison with the amino acid sequence of coronaviral spike (S) proteins resulted in the identification of two interesting domains in the C-terminal part of the BEV polypeptide. At positions 1158-1230 and 1504-1545 in the predicted protein sequence a seven-residue periodicity was identified in which hydrophobic amino acids are present at every first and fourth position (Fig. 6). These so-called "heptad repeats" are indicative of a coiled-coil protein structure. In this conformation  $\alpha$ -helical domains are stabilized by the interaction of regularly spaced hydrophobic residues which form the interface between two  $\alpha$ -helices (Cohen and Parry, 1986).

As in the coronaviral S protein, the two heptad repeats in the BEV P protein are of unequal length, which leaves a part of the major helix unpaired. It has been



Fig. 6. Heptad repeat patterns in the sequence of the BEV peplomer protein. The sequence is listed vertically in alternating rows of three and four residues. Regularly spaced hydrophobic residues (in the a and d positions of the heptads; see panel C) are boxed. In the proposed coiled-coil structure these residues are predicted to form a hydrophobic interface, which interacts with similar regions of other  $\alpha$ -helices. The numbers of the first and last amino acids of the large (A) and small (B) heptad repeat domains are indicated.

postulated that the major heptad repeat in the coronaviral protein is involved not only in intra-chain coiledcoiling, but also in inter-chain interactions (De Groot *et al.*, 1987), which could play a role in the previously reported oligomerization of the coronaviral S protein (Cavanagh, 1983).

The quaternary structure of the BEV P protein was studied by the sucrose gradient assay which has been employed to establish the oligomeric nature of the influenza virus hemagglutinin and the vesicular stomatitis virus G protein (Doms and Helenius, 1986; Doms *et al.*, 1988). TX-100 lysates of BEV-infected cells were subjected to centrifugation in a linear sucrose gradient to separate monomeric and oligomeric forms of the BEV P protein. A set of four sedimentation markers was used for comparison: apoferritin (an oligomer with  $M_r$  443K; Chrichton *et al.*, 1973), catalase (a tetramer with  $M_r$  232K; Schroeder *et al.*, 1969), lactate dehydrogenase (a tetramer with  $M_r$  137K; Huston *et al.*, 1972), and albumin ( $M_r$  67K; Squire *et al.*, 1968).

After gradient fractionation and immunoprecipitation, most of the P protein precursor was recovered from fraction 7 (Fig. 7), sedimenting slightly slower than ferritin (443K; fraction 6) and considerably faster than catalase (232K; fraction 11). These experiments clearly show that most of the precursor in infected cells is present in a dimeric form. Gradient fraction 7 also contained the characteristic heterogeneous peplomer material of 75K–100K which is present in mature virions. A small amount of monomeric precursor was recovered from fractions 13 and 14. The  $M_r$  of this material was slightly higher than the  $M_r$  of the precursor in the dimer peak. In addition, fractions 12 to 14 contained two bands of cleaved and possibly immature peplomer material in the 75K–100K region. On the basis of their sedimentation behavior, it can be concluded that these proteins are also in a dimeric state. Since the peaks of the 75K and the 100K species do not coincide, we assume that this material consists of homodimers of C- and N-terminal P protein subunits, respectively.

#### DISCUSSION

In this paper we report the identification and sequence of the BEV peplomer protein gene. An ORF of 4.7 kb which is located at the 5' end of mRNA 2 encodes a 178K precursor protein. Antibodies directed against the virion P protein specifically immunoprecipitated a 190K protein from lysates of cells which were transfected with a T7 expression vector containing the full-length 4.7-kb ORF. The P protein displays several features which are common to membrane-associated proteins. An N-terminal signal sequence, a possible Cterminal transmembrane anchor, and a number of potential N-glycosylation sites were identified in the deduced amino acid sequence. Furthermore, a candidate cleavage site in the P protein precursor was identified. A host-dependent "trypsin-like" cleavage occurs during the maturation of the envelope proteins of viruses in a number of families. The cleavage site usually is a domain containing multiple basic amino acid residues (Cavanagh et al., 1986). Removal of oligosaccharides (Fig. 5) from the mature P protein showed that the Cterminal P protein subunit contains a polypeptide backbone of the size predicted from the putative cleavage site.

Electron microscopy has revealed projections on the BEV surface, which consist of a globular structure on top of an elongated stem (Weiss *et al.*, 1983). A coiled-coil supersecondary structure, as it has been found for other viral (Wilson *et al.*, 1981; De Groot *et al.*, 1987) and non-viral (Metcalf *et al.*, 1987) surface proteins, can explain the elongated shape of the BEV peplomer. For the two heptad repeat domains in the C-terminal subunit (Fig. 6)  $\alpha$ -helices of about 10 and 6 nm are predicted. In an intra-chain coiled-coil 4 nm of the major helix would remain unpaired; as postulated for its coronaviral equivalent (De Groot *et al.*, 1987), the stability of an oligomeric peplomer complex could be enhanced by the formation of inter-chain coiled-coils.

The oligomerization assay described in this paper proves that the BEV P protein in infected cells is predominantly present in a dimeric form (Fig. 7). Similar experiments in our laboratory have confirmed the dimeric nature of the coronaviral S protein (P. J. M. Rottier *et al.*, personal communication). Heptad repeats are

FIG. 7. Analysis of BEV P protein oligomerization. TX-100-lysed BEV-infected EMS cells were subjected to centrifugation in a 5–20% linear sucrose gradient. After fractionation from the bottom, the BEV P protein was immunoprecipitated with a rabbit antiserum. A set of four sedimentation markers was included for comparison; their position in the gradient is indicated: FER, apoferritin,  $M_r$  443K; CAT, catalase,  $M_r$  232K; LDH, lactate dehydrogenase,  $M_r$  137K; BSA, bovine serum albumin,  $M_r$  67K. Most P protein material was precipitated from fraction 7, corresponding to an  $M_r$  of about 400K. This illustrates the dimeric nature of the BEV P protein precursor in infected cells.

probably not the only domains which are involved in oligomerization, but the predicted intra- and inter-chain interactions are likely to stabilize the characteristic surface projections of both toro- and coronaviruses.

Intracellular transport from ER to medial Golgi and cleavage of the BEV P protein in infected cells seem to be quite slow: after a  $3\frac{1}{2}$ -hr chase, it was estimated that 40-50% of the label incorporated into the P protein was still present in uncleaved endo H-sensitive precursor material. Preliminary data on the processing of the recombinant P protein indicate that its maturation may be even slower in the absence of virus assembly: after a 5-hr chase no P protein cleavage products could be detected in pTP-transfected Hela cells (data not shown). Recently, the transport of the coronaviral S protein has been shown to be significantly slower in the absence of virus assembly: in coronavirus-infected cells the S protein acquires endo H-resistance more rapidly than in cells expressing only the recombinant S protein (De Groot et al., 1989; Vennema et al., 1990). Like coronaviruses, toroviruses have been shown to bud intracellularly, predominantly at the membranes of the Golgi system (Weiss and Horzinek, 1986; Fagerland et al., 1986). Retardation of the BEV P protein in premedial Golgi compartments may therefore promote efficient virus budding (Vennema et al., 1990).

The BEV P protein resembles the coronaviral S protein not only because of the kinetics of its intracellular transport. Both are *N*-glycosylated proteins of about the same size which contain a "trypsin-like" cleavage site (not in all coronaviruses), heptad repeats, and hydrophobic domains in comparable positions in the sequence. In addition, the electron microscopical image and the formation of dimers indicate that toro- and coronaviral spikes are also likely to have similar tertiary and quaternary structures. Within the coronavirus family the amino acid sequence of especially the C-terminal half of the spike protein is highly conserved (De Groot et al., 1987). Since no significant sequence similarities between BEV P and coronaviral S protein sequences were identified, convergent evolution could be invoked to explain the comparable organization and structure of the BEV P protein. However, the recent discovery of a number of conserved domains in the polymerase genes of the coronaviruses and the torovirus BEV indicates that both virus groups are evolutionarily related (Snijder et al., 1990b). Hence, we propose that the toro- and coronaviral spikes are homologous and not analogous structures. The absence of antigenic relationships and amino acid homologies among their surface proteins is indicative for the large evolutionary distance between both virus groups.

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#### REFERENCES

- CAVANAGH, D. (1983). Coronavirus IBV: Structural characterization of the spike protein. J. Gen. Virol. 64, 2577–2583.
- CAVANAGH, D., DAVIS, P. J., PAPPIN, D. J. C., BINNS, M. M., BOURSNELL, M. E. G., and BROWN, T. D. K. (1986). Coronavirus IBV: Partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg–Arg–Phe–Arg–Arg at the cleavage site of the spike

precursor polypeptide of IBV strains Beaudette and M41. *Virus Res.* **4**, 133–143.

- COHEN, C., and PARRY, D. A. D. (1986). α-Helical coiled coils—A widespread motif in proteins. *Trends. Biochem. Sci.* **11**, 245–248.
- CHRICHTON, R. R., EASON, R. BARCLAY, A., and BRYCE, C. F. A. (1973). The subunit structure of horse spleen apoferritin: The molecular weight of the oligomer and its stability to dissociation by dilution. *Biochem. J.* **131**, 855–857.
- DE GROOT, R. J., LUYTJES, W., HORZINEK, M. C., VAN DER ZEIJST, B. A. M., SPAAN, W. J. M., and LENSTRA, J. A. (1987). Evidence for a coiled-coil structure in the spike proteins of coronaviruses. *J. Mol. Biol.* **196**, 963–966.
- DE GROOT, R. J., VAN LEEN, R. W., DALDERUP, M. J. M., VENNEMA, H., HORZINEK, M. C., and SPAAN, W. J. M. (1989). Stably expressed FIPV peplomer protein induces cell fusion and elicits neutralizing antibodies in mice. *Virology* **171**, 493–502.
- DOMS, R. W., and HELENIUS, A. (1986). Quaternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* **60**, 833– 839.
- DOMS, R. W., RUUSALA, A., MACHAMER, C., HELENIUS, J., HELENIUS, A., and ROSE, J. K. (1988). Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. 107, 89–99.
- DUNPHY, W. G., and ROTHMAN, J. E. (1985). Compartmental organization of the Golgi stack. *Cell* **42**, 13–21.
- ELDER, J. H., and ALEXANDER, S. (1982). Endo-β-N-acetylglucosaminidase F: Endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA* **79**, 4540–4544.
- FAGERLAND, J. A., POHLENZ, J. F. L., and WOODE, G. N. (1986). A morphological study of the replication of Breda virus (proposed family *Toroviridae*) in bovine intestinal cells. J. Gen. Virol. 67, 1293–1304.
- FUERST, T. R., NILES, E. G., STUDIER, F. W., and MOSS, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83, 8122–8126.
- GORMAN, C. (1985). High efficiency gene transfer into mammalian cells. *In* "DNA Cloning," Vol. II, "A Practical Approach" (D. M. Glover, Ed.), pp. 143–165. IRL Press, Oxford, UK.
- HORZINEK, M. C., and WEISS, M. (1984). Toroviridae: A taxonomic proposal. Zentralbl. Veterinaer. Med. Reihe B 31, 649–659.
- HORZINEK, M. C., WEISS, M., and EDERVEEN, J. (1984). Berne virus is not 'coronavirus-like'. J. Gen. Virol. 65, 645–649.
- HORZINEK, M. C., EDERVEEN, J., KAEFFER, B., DE BOER, D., and WEISS, M. (1986). The pepiomers of Berne virus. *J. Gen. Virol.* **67**, 2475– 2483.
- HORZINEK, M. C., FLEWETT, T. H., SAIF, L. J., SPAAN, W. J. M., WEISS, M., and WOODE, G. N. (1987). A new family of vertebrate viruses: *Toroviridae*. Intervirology **27**, 17–24.
- HUSTON, J. S., FISH, W. W., MANN, K. G., and TANFORD, C. (1972). Studies on the subunit molecular weight of beef heart lactate dehydrogenase. *Biochemistry* 11, 1609–1612.

KAEFFER, B., VAN KOOTEN, P., EDERVEEN, J., VAN EDEN, W., and HORZI-

NEK, M. C. (1989). Properties of monoclonal antibodies against Berne virus (*Toroviridae*). *Amer. J. Vet. Res.* **50**, 1131–1137.

- KOZAK, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15, 8125–8148.
- KYTE, J., and DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105-132.
- LIPMAN, D. J., and PEARSON, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.
- METCALF, P., BLUM, M., FREYMANN, D., TURNER, M., and WILEY, D. C. (1987). Two variant surface glycoproteins of *Trypanosoma brucei* of different sequence classes have similar 6 Å resolution X-ray structure. *Nature (London)* **325**, 84–86.
- SCHROEDER, W. A., SHELTON, J. R., SHELTON, J. B., ROBBERSON, B., and APELL, G. (1969). The amino acid sequence of bovine liver catalase: A preliminary report. Arch. Biochem. Biophys. 131, 653–655.
- SNIJDER, E. J., EDERVEEN, J., SPAAN, W. J. M., WEISS, M., and HORZINEK, M. C. (1988). Characterization of Berne virus genomic and messenger RNAs. J. Gen. Virol. 69, 2135–2144.
- SNUDER, E. J., HORZINEK, M. C., and SPAAN, W. J. M. (1990a). A 3'coterminal nested set of independently transcribed messenger RNAs is generated during Berne virus replication. J. Virol. 64, 331 – 338.
- SNUDER, E. J., DEN BOON, J. A., BREDENBEEK, P. J., HORZINEK, M. C., RUNBRAND, R., and SPAAN, W. J. M. (1990b). The carboxyl-terminal part of the putative Berne virus polymerase is expressed by ribosomal frameshifting and contains sequence motifs which indicate that toro- and coronaviruses are evolutionarily related. *Nucleic Acids Res.* 18, in press.
- SQUIRE, P. G., MOSER, P., and O'KONSKI, C. T. (1968). The hydrodynamic properties of bovine serum albumin monomer and dimer. *Biochemistry* 7, 4261–4272.
- STADEN, R. (1982). An interactive graphics program for comparing and aligning nucleic acid and amino acid sequences. *Nucleic Acids. Res.* **10**, 2951–2961.
- STADEN, R. (1986). The current status and portability of our sequence handling software. *Nucleic Acids Res.* 14, 217–233.
- TARENTINO, A. L., and MALEY, F. (1974). Purification and properties of an endo-β-N-acetylglucosaminidase from Streptomyces griseus. J. Biol. Chem. 249, 811-817.
- VENNEMA, H., HEUNEN, L., ZUDERVELD, A., HORZINEK, M. C., and SPAAN, W. J. M. (1990). Intracellular transport of recombinant coronavirus spike proteins: Implications for virus assembly. *J. Virol.* 64, 339– 346.
- VON HEUNE, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14, 4683–4690.
- WEISS, M., STECK, F., and HORZINEK, M. C. (1983). Purification and partial characterization of a new enveloped RNA virus (Berne virus). J. Gen. Virol. 64, 1849–1858.
- WEISS, M., and HORZINEK, M. C. (1986). Morphogenesis of Berne virus (proposed family *Toroviridae*). J. Gen. Virol. 67, 1305–1314.
- WILSON, I. A., SKEHEL, J. J., and WILEY, D. C. (1981). Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (London)* 289, 366–373.