EQUINE ARTERITIS VIRUS CONTAINS A UNIQUE SET OF FOUR STRUCTURAL PROTEINS

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INTRODUCTION

Equine arteritis virus (EAV) belongs to a group of small enveloped positive-stranded RNA viruses, provisionally designated arteriviruses, which further includes lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and swine infertility and respiratory syndrome virus (SIRSV) or Lelystad virus (LV). These viruses resemble togaviruses in their physicochemical properties, the polarity and size (13-15 kb) of their genomes, and the size (50-70 nm) and morphology (isometric) of the virions[1,2] but virus assembly occurs by intracellular budding[1,3]. In contrast, the genome organization and gene expression strategy are similar to those of corona- and toroviruses[2,4,5,6,7,8].

The 5' three-fourths of the arterivirus genome consists of two overlapping open reading frames (ORFs 1a and 1b) which are translated from the genomic RNA (RNA 1) and encode the viral replicase. ORF 1b is expressed by ribosomal frameshifting and possesses four amino acid domains characteristic for the polymerases of corona- and toroviruses[2,4,6]. The 3' part of the arterivirus genome contains six overlapping ORFs expressed from a 3' coterminal nested set of subgenomic mRNAs (RNAs 2 to 7). The subgenomic mRNAs possess a common leader sequence derived from the 5' end of the viral genome. The leader sequence is joined to the coding parts of the mRNAs at conserved sequence motifs located upstream of ORFs 2 to 7[2,5,7]. The identity of the expression products of ORFs 2 to 7 has not yet been established except for LDV ORF 7 which encodes the nucleocapsid protein[9].

To further elucidate the evolutionary relationship of arteriviruses with corona- and toroviruses we have studied the structural proteins of EAV and identified the corresponding genes.

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MATERIALS AND METHODS

Cells and viruses

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. A stock of the Bucyrus strain of EAV[10] was prepared in BHK-21 cells. A recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase gene (VTF7-3) was obtained from Dr. B. Moss.

Plasmid construction

ORF 6 was cloned behind a T7 promoter by insertion of a 556 bp *Cla*I-*Fsp*I fragment from cDNA clone 106[5] into *Cla*I- and *Sma*I-digested pBluescript SK(-). The resulting plasmid was designated pAVI16. ORF 5 was placed downstream of a T7 promoter by ligating a 1243 bp *Dra*I-*Hin*dIII fragment from cDNA clone 008[5] into *Sma*I- and *Hin*dIII-digested pBS(+) to yield plasmid pAVI05. Subsequently, plasmid pAVI15 was constructed by cloning the 0.9kb *Sac*I-*Xba*I fragment from pAVI05 into the polylinker region of pBluescript KS(+).

Preparation of antisera

Antisera directed against the carboxy termini of the ORF 7, 6, and 2 translation products were produced by subcutaneous injection of rabbits at monthly intervals with synthetic peptides SP07 (NH_2-Tyr-Trp-Val-Pro-Thr-Lys-Gln-Ile-Gln-Arg-Lys-Val-Ala-Pro-Pro-Ala-Gly-Pro-COOH), SP06 (NH_2-Tyr-Ala-Gly-Arg-Leu-Phe-Ser-Lys-Arg-Thr-Ala-Ala-Thr-Ala-Tyr-Lys-Leu-Gln-COOH), and SP02 (NH_2-Cys-Pro-Ser-Arg-Arg-Thr-Ser-Ser-Gly-Thr-Leu-Pro-Arg-Arg-Lys-Ile-Leu-COOH), respectively. The peptides were coupled to keyhole limpet hemocyanin prior to immunization. A serum directed against the structural proteins of EAV was prepared by multiple inoculations of a rabbit with sucrose gradient purified virus. For the primary immunizations the antigens were emulsified in Freund's complete adjuvant, for the booster injections Freund's incomplete adjuvant was used.

Expression of ORF 5 and 6

BHK-21 cells were transfected with plasmid pAVI16 or pAVI15 using cationic liposomes[11]. After incubation for 5 h at 37°C, the cells were infected at a MOI of 10 with the vaccinia virus recombinant vTF7-3 expressing the bacteriophage T7 RNA polymerase[12]. Metabolic labeling was started at 10 h after transfection. The labeling regimen and cell lysis procedure were as described for EAV-infected BHK-21 cells.

Radioactive labeling of intracellular proteins

Subconfluent monolayers of BHK-21 cells were infected with EAV at a MOI ≥ 20 as described previously[13]. The cells were starved in methionine free medium for 30 min at 7½ h p.i. and subsequently labelled for 20 min at 37°C by addition of 100 µCi/ml Tran[^{35}S]-label (> 1000 Ci/mmol; ICN). RK-13, LLC-MK2, and VERO C1008 cells were similarly labelled at 6, 8, and 12 h p.i., respectively. In some experiments a 2 h chase was performed with DMEM-10% FCS-2 mM methionine. The cells were lysed in 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) containing

1 µg/ml of aprotinin, leupeptin, and pepstatin A. The lysates were cleared by centrifugation and supplemented with EDTA to a 5 mM final concentration.

Preparation of radiolabeled virions

Subconfluent monolayers of BHK-21 cells were infected with EAV at a high MOI. After incubation for 7 h at 39.5°C the medium was replaced by methionine-free or cysteine-free medium containing 2% FCS and 100 µCi/ml L-[^{35}S]methionine or L-[^{35}S]cysteine (> 1000 Ci/mmol; ICN), respectively. At 10½ h p.i. one fifth a volume of DMEM-10% FCS was added and the cells were further incubated for 3½ h at 39.5°C. Finally, an excess methionine or cysteine was added and the incubation was continued for 30 min. The medium was then collected and the virus was purified by PEG precipitation followed by sucrose gradient fractionation[13].

Immunoprecipitation and gel electrophoresis

Protein samples were diluted in immunoprecipitation buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% sodium deoxycholate, 1 µg/ml protease inhibitors) containing either 0.1% SDS (anti-virion and anti-SP07 serum) or 0.25% SDS (anti-SP06 and anti-SP02 serum); 3 µl rabbit serum was added and the samples were incubated overnight at 4°C. The immune complexes were adsorbed to Pansorbin^{R} (Calbiochem) and collected by centrifugation. Pellets were washed three times in 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40 and once in 20 mM Tris-HCl (pH 7.6), 0.1% NP-40. The immune complexes were dissolved in 25 µl Laemmli sample buffer and analyzed in SDS-15% polyacrylamide gels[14]. Alternatively, the immunoprecipitates were subjected to endoglycosidase treatment before gel electrophoresis.

Endoglycosidase treatment

Endoglycosidase F/N-glycosidase F (glyco F) (Boehringer Mannheim) digestions were carried out in 50 mM sodium phosphate (pH 6.8), 20 mM EDTA, 1% NP-40, 0.15% SDS, 1% 2-mercaptoethanol containing 1 µg/ml protease inhibitors. The incubations were done overnight at 30°C with 100 mU enzyme per reaction. Endo-ß-galactosidase (endo ß) (from *Bacteroides fragilis*; Boehringer Mannheim) digestions were performed overnight at 37°C using 2 mU enzyme per reaction in 50 mM sodium acetate (pH 5.75), 200 µg/ml acetylated BSA (Biolabs), 1 µg/ml protease inhibitors.

RESULTS

Identification of the structural proteins

To define the structural proteins of EAV, [^{35}S]methionine labeled virus was isolated from the culture medium of EAV-infected BHK-21 cells and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). A set of four virion proteins with molecular weights of 14, 16, 25, and 30 to 42 K was consistently observed (Fig. 1). The 14-, 16-, and 30- to 42-kDa proteins were recognized by an antiserum directed against sucrose gradient purified virus (Fig. 1). Glyco F treatment of radiolabeled virions demonstrated that the two largest proteins are N-glycosylated and confirmed the presence of four structural proteins (data not shown).

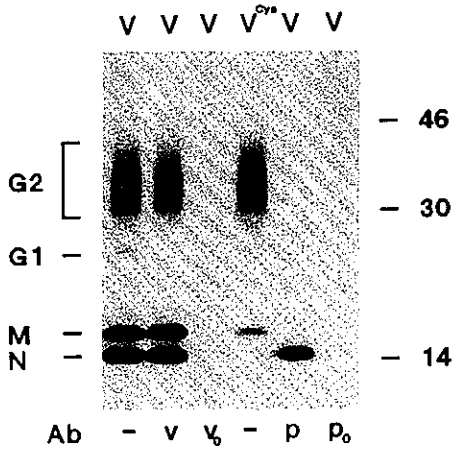

Figure 1. Protein composition of EAV and identification of the ORF 7 product. [^{35}S]methionine (V) and [^{35}S]cysteine (V^{Cys}) labeled virus were analyzed by SDS-PAGE directly or after immunoprecipitation with antibodies (Ab) directed against purified virus (v) or a peptide specific for the ORF 7 protein (p). The corresponding presera (v_0, p_0) did not recognize any of the virion proteins. The positions of the 14-kDa protein (N), the 16-kDa protein (M) and the 25-kDa protein (G_S) are indicated; the bracket depicts the size limits of the heterogeneously glycosylated 30- to 42-kDa protein (G_L). The position and size (in kDa) of marker proteins analyzed in the same gel are indicated at the right.

ORF 7 encodes the 14 kDa nucleocapsid (N) protein

The nucleocapsid of EAV is composed of a single-stranded, polyadenylated genomic RNA and a 12- to 14-kDa core protein[4,15]. Since ORF 7 encodes a basic protein with a predicted molecular weight of 12.3 K[4] and since the in vitro translation product of RNA 7 comigrated in gel with the core protein[16], it probably is the nucleocapsid gene. To test this hypothesis, we exploited the fact that only ORF 7 specifies a viral protein lacking cysteine[4]. Virions were labelled with [^{35}S]methionine or with [^{35}S]cysteine, purified in sucrose gradients and the protein patterns were compared. The nucleocapsid protein of 14 kDa prominent in [^{35}S]methionine labeled virus was indeed absent in the [^{35}S]cysteine labeled preparation (Fig. 1). Moreover, the [^{35}S]methionine labeled 14-kDa protein was also specifically precipitated using an antiserum directed against the 18 carboxy terminal amino acids of the deduced ORF 7 product (Fig. 1).

ORF 6 encodes the 16 kDa membrane (M) protein

The predicted ORF 6 product has a molecular weight close to 16 K, lacks N-glycosylation sites and contains five methionines and one cysteine. In vitro translation of ORF 6 yielded a protein of the anticipated size that was recognized by the anti-virion serum (data not shown). In combination with its relatively low incorporation of [^{35}S]cysteine (Fig. 1) and its insensitivity to glyco F treatment, these data suggested that the 16-kDa virion protein is encoded by ORF 6.

To prove this assumption, BHK-21 cells were transfected with plasmid pAVI16, which has ORF 6 cloned behind a T7 promoter, and infected with recombinant vaccinia virus vTF7-3 synthesizing the bacteriophage T7 RNA polymerase. The cells were metabolically labeled and the ORF 6 product was precipitated using the anti-

virion serum or a specific anti-peptide serum. A 16-kDa protein indistinguishable from that in the [^{35}S]methionine labeled virus preparation was detected with both antisera but not with the corresponding presera (Fig. 2). The anti-virion serum also recognized a protein of 28 kDa. This protein is probably a dimer of the ORF 6 product formed during the analytical procedures. A strong tendency to aggregate has also been reported for the M protein of coronaviruses which shares many characteristics with the EAV ORF 6 product (see discussion). The inability of the anti-peptide serum to immunoprecipitate the dimer may be attributed to the stringent immunoprecipitation conditions (0.25% SDS) that would prevent complex formation. Alternatively, the anti-peptide serum may fail to precipitate the dimer because aggregation renders the carboxy terminus of the ORF 6 protein inaccessible for antibodies.

To complete the evidence that ORF 6 encodes the 16-kDa virion protein, [^{35}S]methionine labeled virus was subjected to immmunoprecipitation using the anti-peptide serum. The 16-kDa virion protein was clearly recognized by the anti-peptide serum (Fig. 2), but a substantial amount of the 30- to 42-kDa protein was precipitated too. The corresponding preserum did not recognize this 30- to 42-kDa smear. The co-precipitation of the 30- to 42-kDa protein presumably results from a specific association with the 16-kDa protein as it is unlikely that both proteins share a common epitope recognized by the anti-peptide serum. This may also explain why the anti-virion serum only weakly binds the ORF 6 product synthesized in pAVI16-transfected cells.

ORF 5 encodes the heterogeneously glycosylated 30-42 kDa (G_L) protein

The predicted ORF 5 product has a molecular weight of 28.7 K and contains one potential N-glycosylation site[4]. In vitro translation of ORF 5 in the presence of microsomes yielded a 30-kDa protein which was strongly recognized by the anti-virion serum. Its apparent molecular weight was reduced to 27 K after glyco F treat-

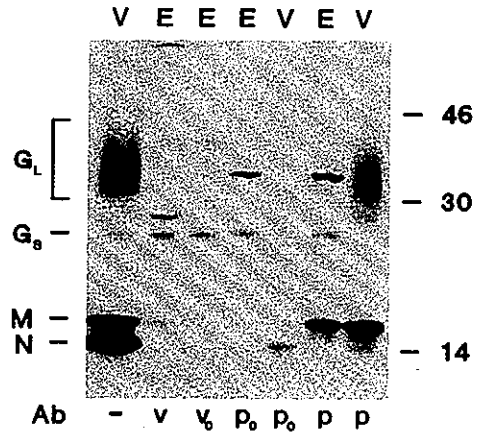

Figure 2. Characterization of the ORF 6 product. BHK-21 cells were transfected with pAVI16, infected with recombinant vaccinia virus vTF7.3 and labeled with [^{35}S]methionine. The ORF 6 product was immunoprecipitated from the cell lysate using the anti-virion serum (v) or a specific anti-peptide serum (p) and the immunoprecipitates were analyzed by SDS-PAGE (lanes E). The protein patterns were compared with those of [^{35}S]methionine labeled virus applied to the gel directly or after immunoprecipitation with the anti-peptide serum (lanes V). The corresponding presera (v_o, p_o) failed to immunoprecipitate the ORF 6 protein from both the transfected cells and the virus preparation.

ment (data not shown). Since the same molecular weight was found for the glyco F digested 30- to 42-kDa virion protein (data not shown) we speculated that ORF 5 encodes the 30- to 42-kDa smear.

To study the ORF 5 expression product, its coding sequence was cloned behind a T7 promoter. The resulting plasmid, designated pAVI15, was expressed in BHK-21 cells which were then labeled for 20 min with Tran[^{35}S]-label. Immunoprecipitates were prepared using the anti-virion serum and treated or mock-treated with glyco F. A discrete 30-kDa product was detected whose molecular weight was reduced to 27 K by the glycosidase (Fig. 3). A 30-kDa protein was also immunoprecipitated from a lysate of EAV-infected cells labeled for the same period. Glyco F digestion converted this protein into a 27-kDa species which comigrated with the deglycosylated form of both the ORF 5 expression product and the 30- to 42-kDa virion protein. To determine whether the 30- to 42-kDa protein derives from the 30-kDa protein by maturation of the N-glycan, we performed a pulse-chase experiment with EAV-infected BHK-21 cells. The 30-kDa protein observed after pulse labeling was processed during a 2 h chase to a 28- to 42-kDa smear (Fig. 3). After treatment with glyco F, the smear was changed into the 27-kDa protein identified before.

To investigate whether the broad size distribution of the 30- to 42-kDa protein is caused by polylactosaminoglycan modification, [^{35}S]methionine labeled virions were treated with endo ß. This enzyme cleaves the internal ß1→4 galactosidic linkages of oligosaccharides with the general sequence R_1-GlcNAcß1→3Galß1→4 GlcNAc/Glc-R_2[17]. Whereas the 25-kDa glycoprotein was resistant to endo ß treatment, the 30- to -42 kDa smear was converted to a discrete band of 30 kDa (Fig. 3). The slight size difference between the glyco F and endo ß treated ORF 5 protein reflects the fact that the latter leaves part of the N-linked oligosaccharide side chain

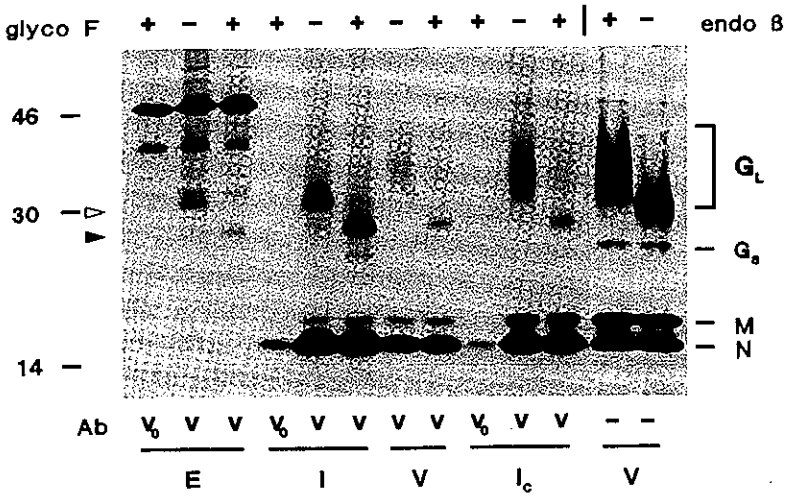

Figure 3. Characterization of the ORF 5 product. BHK-21 cells were transfected with pAVI15, infected with recombinant vaccinia virus vTF7.3 and labeled for 20 min with [^{35}S]methionine (lanes E). EAV-infected BHK-21 cells were labeled with [^{35}S]methionine for the same period (lanes I). Alternatively, the 20 min pulse labeling of EAV-infected cells was followed by a 2 h chase (lanes I_C). The ORF 5 protein was immunoprecipitated from the cell lysates and from a [^{35}S]methionine labeled virus preparation (lanes V) using the anti-virion serum (v). Immunoprecipitations with the corresponding preserum (v_0) were included as a control. The immunoprecipitates were treated (+) or mock-treated (-) with glyco F and analyzed by SDS-PAGE. The position of the glycosylated ORF 5 product obtained after a 20 min pulse is indicated by an open arrowhead. The closed arrowhead points to the glyco F treated ORF 5 protein. The two lanes on the right display [^{35}S]methionine labeled virus treated (+) or mock-treated (-) with endo ß.

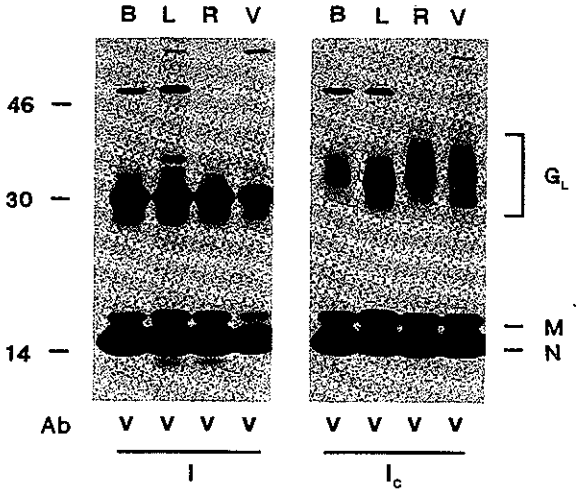

Figure 4. Maturation of the ORF 5 protein in different cell lines. EAV-infected BHK-21 (B), LLC-MK2 (L), RK-13 (R), and VERO C1008 (V) cells were labelled with [^{35}S]methionine for 20 min (I) and chased for 2 h (I_C). The ORF 5 protein was imunoprecipitated from the cell lysates with the anti-virion serum (v) and analyzed by SDS-PAGE.

intact. Since the nature and extent of the polylactosaminoglycan modification of proteins is determined by the particular cell line in which it is synthesized[18,19], we infected four different cell types with EAV and analyzed the ORF 5 products. After pulse labeling for 20 min a single protein of 30 kDa was detected with each cell line. During a 2 h chase the 30-kDa protein was converted into a smear whose appearance in gel was different for distinct cell types (Fig. 4). The heterogeneity in N-glycosylation of the ORF 5 protein was also found in the virions produced by these cell lines (data not shown).

Significantly more of the ORF 6 protein was immunoprecipitated after a 2 h chase than after a pulse (Fig. 3 and 4). Efficient precipitation of the ORF 6 protein by the anti-virion serum may rely upon its interaction with the ORF 5 protein; complex formation between these two may require (partial) maturation of the ORF 5 and/or ORF 6 protein. The failure of the ORF 6 specific anti-peptide serum to recognize the expression product of ORF 5 (data not shown) together with its ability to co-precipitate a substantial portion of the ORF 5 protein from a virus preparation (Fig. 2) provides further evidence for an interaction between the ORF 5 and 6 proteins.

ORF 2 encodes the glycosylated 25 kDa (G_S) protein

The deduced ORF 2 product possesses one possible N-glycosylation site and has a calculated molecular weight of 22.7 K after removal of the putative amino terminal signal sequence[4]. In vitro translation of ORF 2 in the presence of microsomes yielded a product which comigrated with the 25-kDa virion protein. Since the molecular weight of both the 25-kDa virion protein and the translation product of ORF 2 was decreased to 22 K by glyco F treatment (data not shown), ORF 2 was thought to encode the 25-kDa virion protein. For this reason, an anti-peptide serum was raised against the predicted carboxy terminus of the ORF 2 product. The antiserum was applied to a preparation of [^{35}S]methionine labeled

virions. The resulting immunoprecipitate was divided in two portions one of which was treated with glyco F. The anti-peptide serum specifically recognized the 25-kDa virion protein which was converted to a protein of 22 kDa by glyco F (Fig. 4).

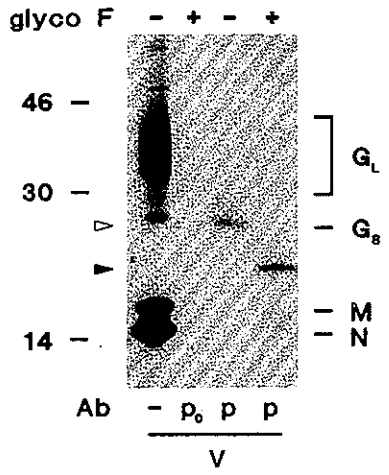

Figure 5. Characterization of the ORF 2 product. The ORF 2 protein was immunoprecipitated from a [^{35}S]methionine labeled virus preparation (lanes V) with a specific anti-peptide serum (p). An immunoprecipitation with the corresponding preserum (p_0) was carried out as a control. The immunoprecipitates were treated (+) or mock-treated (-) with glyco F and analyzed by SDS-PAGE together with [^{35}S]methionine labeled virus. The open arrowhead marks the position of the glycosylated ORF 2 product, the closed arrowhead points to the ORF 2 product obtained after digestion with glyco F.

DISCUSSION

In this paper we showed that the protein composition of EAV is entirely different from that of corona- and toroviruses. From the combination of our results with previous data[15] it follows that virions contain a 14-kDa nucleocapsid protein (N) and three envelope proteins designated M, G_S, and G_L. M is an unglycosylated protein of 16 kDa, G_S and G_L are N-glycosylated proteins of 25 kDa and 30 to 42 kDa, respectively. N, M, and G_L are major structural proteins, G_S is only a minor virion constituent. Although both glycoproteins contain a single N-glycan and pass the same intracellular compartments as part of a virion, maturation of their N-linked oligosaccharide side chains is different. The mature G_L protein is susceptible to digestion with endo-ß-galactosidase which indicates that it becomes glycosylated by the addition of variable numbers of lactosamine (Galß1→4GlcNAcß1→3) repeats to the N-linked core oligosaccharide. G_L is modified in a distinct way in different cell lines as is the case for other proteins that acquire polylactosaminoglycans[18,19]. The extracellular G_S protein is resistant to treatment with endo-ß-galactosidase and endoglycosidase H (data not shown) and therefore probably acquires a regular complex type N-glycan. To date the only other viral proteins known to acquire a polylactosaminoglycan are the NB protein of influenza B virus[18] and the SH proteins of human and bovine respiratory syncytial viruses[19]. The function of the poly-N-acetyllactosamine modification attached to these proteins is unknown.

We also identified the genes for the structural proteins of EAV and compared them with those of corona- and toroviruses. ORF 7 codes for N, ORF 6 for M, ORF 5 for G_L, and ORF 2 for G_S. No amino acid sequence similarities between the

structural protein genes of EAV and those of corona- and toroviruses were observed. However, the hydrophobicity plot of the EAV ORF 6 protein appears to be very similar to that of the membrane protein (M) of coronaviruses[20] and of the envelope protein (E) of toroviruses[21], which suggests that it is also a type III integral membrane proteins containing three successive transmembrane helices. Since the EAV ORF 6 protein accumulates in intracellular membranes too (data not shown), we anticipate that the M (E) proteins of arteri-, corona- and toroviruses are functional homologs. Moreover, the positions of the structural protein genes in the EAV genome correspond with a corona- and toroviruslike gene order: 5'-replicase-envelope glycoprotein(s)-membrane protein (M)-nucleocapsid protein (N)-3'.

The collective data illustrate that arteriviruses resemble corona- and toroviruses in their replication and transcription mechanism although the structural proteins are largely different from those of corona- and toroviruses. The universal presence of a triple-spanning membrane protein among the nested set viruses may relate to their specific mode of intracellular budding. A detailed analysis of the virion structure of arteriviruses and the properties of their structural proteins may uncover yet unsuspected similarities with corona- and toroviruses.

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