Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

VIROLOGY 206, 155–163 (1995)

Characterization of Proteins Encoded by ORFs 2 to 7 of Lelystad Virus

JANNEKE J. M. MEULENBERG,[1] ANNELIEN PETERSEN-DEN BESTEN, ERIC P. DE KLUYVER, ROB J. M. MOORMANN, WIM M. M. SCHAAPER,* AND GERT WENSVOORT

*Department of Virology, Institute for Animal Science and Health (ID-DLO), Houtribweg 39, NL-8200 AJ Lelystad, The Netherlands; and *Laboratory of Molecular Immunology, Institute for Animal Science and Health (ID-DLO), Edelhertweg 15, NL-8200 AB Lelystad, The Netherlands*

Received July 5, 1994; accepted September 20, 1994

The genome of Lelystad virus (LV), a positive-strand RNA virus, is 15 kb in length and contains 8 open reading frames (ORFs) that encode putative viral proteins. ORFs 2 to 7 were cloned in plasmids downstream of the Sp6 RNA polymerase promoter, and the translation of transcripts generated *in vitro* yielded proteins that could be immunoprecipitated with porcine anti-LV serum. Synthetic polypeptides of 15 to 17 amino acids were selected from the amino acid sequences of ORFs 2 to 7 and antipeptide sera were raised in rabbits. Antisera that immunoprecipitated the *in vitro* translation products of ORFs 2 to 5 and 7 were obtained. Sera containing antibodies directed against peptides from ORFs 3 to 7 reacted positively with LV-infected alveolar lung macrophages in the immunoperoxidase monolayer assay. Using these antipeptide sera and porcine anti-LV serum, we identified three structural proteins and assigned their corresponding genes. Virions were found to contain a nucleocapsid protein of 15 kDa (N), an unglycosylated membrane protein of 18 kDa (M), and a glycosylated membrane protein of 25 kDa (E). The N protein is encoded by ORF7, the M protein is encoded by ORF6, and the E protein is encoded by ORF5. The E protein in virus particles contains one or two *N*-glycans that are resistant to endo-**β**-*N*-acetyl-D-glucosaminidase H. This finding indicates that the high-mannose glycans are processed into complex glycans in the Golgi compartment. The protein composition of the LV virions further confirms that LV is evolutionarily related to equine arteritis virus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus.

INTRODUCTION

Lelystad virus (LV) is a small enveloped virus containing a positive-strand RNA genome. It was first identified in 1991 in The Netherlands by Wensvoort *et al.* (1991) and in the United States by Collins *et al.* (1992) as the causative agent of porcine reproductive respiratory syndrome (PRRS). PRRS is mainly characterized by reproductive failure in sows and respiratory problems in pigs of all ages (Loula, 1990; Wensvoort, 1993).

The genome of LV is a polyadenylated RNA molecule of about 15 kb, which contains eight open reading frames (ORFs) that probably encode the replicase genes (ORFs 1a and 1b), the envelope proteins (ORFs 2 to 6) and the nucleocapsid protein (ORF7; Meulenberg *et al.*, 1993a; Conzelmann *et al.*, 1993). ORFs 2 to 7 are most likely expressed from six subgenomic RNAs, which are synthesized during replication (Meulenberg *et al.*, 1993a). These subgenomic RNAs form a 3′ coterminal nested set and are composed of a common leader, derived from the 5′ end of the viral genome which is joined to the body of the subgenomic RNAs at the consensus junction sequence UCAACC (Meulenberg *et al.*, 1993b).

LV (also named PRRS virus) resembles equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) in genome organization, replication strategy, amino acid sequence of the proteins, and preference for infection of macrophages, both *in vivo* and *in vitro* (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993a). Because of these similarities, proposals have been made to classify LV, EAV, SHFV, and LDV into a new virus family, tentatively named the *Arteriviridae* (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993a; Plagemann and Moennig, 1991).

When isolates of LV from Europe and the United States were tested in an immunoperoxidase monolayer assay using polyclonal sera containing antibodies against these isolates, they appeared to differ antigenically, but to share common antigens (Wensvoort *et al.*, 1992). Murtaugh *et al.* (1993) also observed genomic heterogeneity between the nucleotide sequence of a United States isolate and LV. Apparently, these viruses have diverged from each other by point mutation and possibly through recombination.

Although the replication strategy of LV has been studied, and the complete nucleotide sequence of the viral genome has been determined, little is still known about the structural proteins of LV. Using hyperimmune pig antisera and monoclonal antibodies, Nelson *et al.* (1993) have identified three viral proteins of 15, 19, and 26-kDa in lysates of cells infected with a United States isolate of LV. They considered the 15-kDa protein to be the nucleocapsid protein. In the work described in this paper

[1] To whom correspondence and reprint requests should be addressed. Fax: 31-320042804.

0042-6822/95 $6.00

the viral proteins of LV were studied in more detail. The proteins encoded by ORFs 2 to 7 were characterized by *in vitro* transcription and translation experiments; proteins encoded by ORFs 5 to 7 were shown to be structural proteins of LV.

MATERIALS AND METHODS

Cells and viruses

LV was grown either on porcine alveolar macrophages or on CL2621 cells (courtesy of Boehringer-Ingelheim, St. Joseph, MO). Macrophages were maintained as described before (Wensvoort *et al.*, 1991). CL2621 cells were maintained in Eagle's basal medium supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. To prepare concentrated and purified virions, confluent monolayers of CL2621 cells were infected at a multiplicity of infection (m.o.i.) of 0.1. At the beginning of cytopathic changes (48–56 hr after infection), the medium was harvested and centrifuged for 20 min at 1200 *g*. The virus in the medium was concentrated by precipitating it with 6% polyethylene glycol 20,000 overnight at 4° and was then centrifuged at 10,000 *g* for 45 min. The pellet was resuspended in TNE buffer (0.01 *M* Tris–HCl, pH 7.2, 0.1 *M* NaCl, 1 m*M* EDTA) and layered either on a 30–0% glycerol, 0–50% di-K-tartrate gradient (Purchio *et al.*, 1984) or on a 20–50% sucrose gradient (van Berlo *et al.*, 1982).

Computer analysis of amino acid sequences

To identify specific characteristics in the amino acid sequences of ORFs 2 to 7, the computer analysis program PCGENE (Intelligenetics, Inc., Mountain View, CA) was used. Prosite was used to detect sites in the protein sequence that might have a putative specific biological function (Bairoch *et al.*, 1991). Hydrophobic membrane-spanning sequences and signal sequences were identified by the methods of Kyte and Doolittle (1982) and von Heijne (1986), respectively.

Plasmid constructions

Specific oligonucleotides located upstream and downstream of ORFs 2 to 7 were synthesized. These were 5′ CTGCCGCCCGGGCAAGTGCC 3′ and 5′ AGAACAGGGCATGCATATGG 3′ for ORF2, 5′ CACGCCAGGTACCAGGCCCA 3′ and 5′ AAAGCATCTGCAGGTCCGCG 3′ for ORF3, 5′ GGCAATTGGATCCATTTGGA 3′ and 5′ AGAAGCAAGCTTGCGGAGTC 3′ for ORF4, 5′ GAGGTGGGATCCAACCATTG 3′ and 5′ CTAGCACAAGCTTTTGTGCG 3′ for ORF5, and 5′ CCCTTGACGAGCTCTTCGGC 3′ and 5′ CCATCGGATCCGTACTTTTC 3′ for ORF6, and 5′ GGTTAACCTCGTCGACTATG 3′ and 5′ CCTGATTAAGCTTGACCCCC 3′ for ORF7. The underlined nucleotides are mutated with respect to the genome sequence of LV, in order to create restriction sites in the primers. These primers were used to amplify ORFs 2 to 7 by the polymerase chain reaction (PCR) from cDNA clones described earlier (Meulenberg *et al.*, 1993a). The ORFs were amplified with AmpliTaq DNA polymerase (Perkin–Elmer–Cetus) under the following conditions: Double-stranded DNA was denatured for 1 min at 94°, the primer was annealed for 1 min at 55° and extended for 1 min at 72°. The number of cycles was 30. The restriction sites incorporated in the primers were used to clone the ORFs downstream of the Sp6 RNA polymerase promoter of pGEM-4Z (Promega). Recombinant DNA techniques were performed essentially as described by Sambrook *et al.* (1989).

In vitro transcription and translation

Plasmids containing ORFs 2 to 7 were linearized and transcribed *in vitro* by use of Sp6 RNA polymerase (Promega), according to the protocol of Promega. The synthesized RNA was translated in rabbit reticulocyte lysate (Promega) in the presence of L-[^{35}S]methionine (>1000 Ci/mmol, Amersham), as recommended by the manufacturer, in either the presence or the absence of microsomal membranes.

Endoglycosidase treatment

In vitro translation products, purified LV preparations, or immunoprecipitates were resuspended in 25 μl endoglycosidase buffer (1% NP-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin in phosphate-buffered saline (PBS)). Then 800 mU of peptide *N*-glycosidase F (PNGaseF; Boehringer-Mannheim) was added and the reaction mixture was incubated overnight at 37°. Controls were treated similarly, except that the PNGaseF was omitted. Endo-β-*N*-acetyl-D-glucosaminidase H (EndoH) digestion was performed similarly, except that the protein samples were resuspended in 100 m*M* sodium acetate (pH 5.5), 1% NP-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 20 mU EndoH (Boehringer-Mannheim). Endo-β-galactosidase digestions were performed as described by de Vries *et al.* (1992).

Preparation of antisera

Polyvalent antiserum 21 directed against LV was obtained from a specific-pathogen-free (SPF) pig infected intranasally with 10^5 $TCID_{50}$ of a fifth cell culture passage of LV (CDI-NL-91; Institute Pasteur I-1102). Blood samples were taken 42 days after infection. Gene-specific rabbit sera directed against ORFs 2 to 7 were obtained by use of synthetic peptides of 15 to 17 residues containing an amino acid sequence specific for each ORF. The amino acid sequences were selected on the basis of hydrophilicity, which was determined by the algorithms of Hopp and Woods (1981) and the antigen prediction

program Asciflex (Janin, 1979; Parker *et al.,* 1986). The peptides were conjugated to keyhole limpet hemocyanin. SPF rabbits were immunized intramuscularly and subcutaneously with 1 mg peptide conjugated to keyhole limpet hemocyanin in complete Freund's adjuvant. After 1 month, the rabbits were given a booster injection of the same amount of conjugated peptide in incomplete Freund's adjuvant. The rabbits were bled at 12 weeks after the first immunization. Sera were tested for their reactivity with the various peptides in an enzyme-linked immunosorbent assay (ELISA), using peptides coated to M96 plates (Suter, 1982). Sera were also tested for their reactivity with viral antigen in an immunoperoxidase monolayer assay (IPMA), essentially as described by Wensvoort *et al.* (1991). Porcine alveolar lung macrophages were seeded in microtiter plates as described and grown for 16 hr. Then 50 μl medium containing 10^3–10^4 $TCID_{50}$ LV was added, and the macrophages were grown for 24 hr. The plates were then fixed and stained with the rabbit antipeptide sera and goat anti-rabbit IgG horseradish peroxidase, as described. As previously established, these incubation times resulted in 30% of the macrophages being infected and showing good cytoplasmic staining in IPMA. Sera 690, 694, 698, 704, 710, and 714 directed against ORFs 2, 3, 4, 5, 6, and 7, respectively, were selected for further use in immunoprecipitation and Western blot analysis of viral proteins produced *in vitro* or *in vivo.* Monoclonal antibodies raised against a United States isolate (SDOW12 and SDOW17) and an English isolate of LV (WBE1, WBE 4 to 6) were obtained from D. Benfield and T. Drew, respectively.

Western blot analysis

Viral protein samples were suspended in Laemmli sample buffer (Laemmli, 1970), heated for 2 min at 100°, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on gels containing 12.5 or 15% polyacrylamide. The separated proteins were transferred to nitrocellulose paper by electroblotting (Towbin *et al.,* 1979). Polyclonal antiserum 21 and antipeptide sera 690, 694, 698, 704, 710, and 714 were diluted 1:50 in PBS containing 2% NaCl, 0.05% Tween 80, and 5% horse serum. Nitrocellulose strips were incubated with these diluted antisera for 1 hr at 37°. The strips were washed three times with PBS containing 2% NaCl and 0.05% Tween 80. They were then incubated with rabbit anti-swine IgG horseradish peroxidase (1:500) or goat anti-rabbit IgG horseradish peroxidase (1:1000) diluted in PBS containing 2% NaCl, 0.05% Tween 80, and 5% horse serum for 1 hr at 37°. Finally, the strips were washed three times in PBS and stained in a solution of 0.6 mg/ml 4-chloro-1-naphthol, 20% (v/v) methanol, and 0.3 μl/ml H_2O_2 (30%).

Radioactive labeling of intracellular proteins

Confluent monolayers of CL2621 were infected with LV as described above, at an m.o.i. of 0.1. At 24 hr after infection, the medium was replaced by methionine- and cysteine-free medium containing 10% fetal calf serum, 90 μCi/ml L-[^{35}S]methionine, and 125 μCi/ml L-[^{35}S]cysteine (>1000 Ci/mmol, Amersham). Cells were harvested 40 hr after infection, rinsed in PBS, and lysed in PBS–TDS (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 m*M* phenylmethylsulfonyl fluoride in PBS). Lysates were sonified and clarified for 10 min at 120,000 *g.* The supernatant was used for immunoprecipitation.

Immunoprecipitation and gel electrophoresis

Proteins were immunoprecipitated essentially according to the method of Hulst *et al.* (1993). *In vitro* translation products were diluted in PBS–TDS and incubated overnight at 4° with 10 μl polyvalent serum 21 or antipeptide serum. The immunocomplexes were then precipitated with protein A–Sepharose CL-4B beads (Pharmacia) for 2 hr at 4°. The protein A–Sepharose beads were pelleted (2 min at 1000 *g*) and were washed four times with PBS–TDS. Pellets were then resuspended in Laemmli sample buffer and heated for 2 min at 100° before analysis by SDS–PAGE on gels containing 12.5 or 15% polyacrylamide. Gels were dried and immunoprecipitated proteins were visualized by autoradiography. The same procedure was used to precipitate proteins from cell lysate or purified virus, except that the samples were first immunoprecipitated with negative pig serum or rabbit preserum to reduce nonspecific precipitation of proteins.

RESULTS

Computer analysis of ORFs 2 to 7 of Lelystad virus

The positive-strand RNA genome of LV is composed of 15,088 bases and contains two large ORFs (ORF1a and ORF1b) encoding the RNA-dependent RNA polymerase and six smaller ORFs (ORFs 2 to 7) located at the 3′ part of the viral genome. ORFs 2 to 7 are 128 to 265 amino acids in length and possibly encode structural proteins of LV (Table 1). These ORFs contain putative N-glycosylation sites, which may mean that they encode glycoproteins. ORFs 2, 3, and 5 contain a hydrophobic amino terminus followed by a putative signal cleavage site as predicted by the method of Von Heijne (1986). These sequences may be involved in the transport of ORFs 2, 3, and 5 to the endoplasmic reticulum. ORF4 also contains an N-terminal hydrophobic sequence, but no signal sequence cleavage site was predicted. Hydrophobic sequences encoded at the carboxy termini of ORFs 2 and 4 and in the middle of the coding region of ORF5 may function as membrane anchors. ORF6 contains three putative membrane-spanning fragments at the N-terminus. The hydrophobicity profile of this protein is comparable to that of the membrane protein M of

TABLE 1

CHARACTERISTICS OF PROTEINS ENCODED BY ORFs 2 TO 7 OF LV

ORF	No. of amino acids	Calculated MW[a]	N-glycosylation sites[b]	Signal sequence[c]	Putative transmembrane region	MW on SDS–PAGE[d]	
						In vitro	*In vivo*
2	249	28.4	2	37–38	210–228	30	—
3	265	30.6	7	41–42	—	45	—
4	183	20.0	4	—	1–17/165–183	31	—
5	201	22.4	2	32–33	108–131	25	25
6	173	18.9	2	—	17–88	18	18
7	128	13.8	1	—	—	15	15

[a] Calculated MW (kDa) on the basis of the amino acid sequence.
[b] Number of putative N-glycosylation sites.
[c] Amino acid position of the putative signal sequence cleavage site.
[d] MW (kDa) on SDS–PAGE of the gene products of the ORFs expressed *in vitro* or *in vivo*.

coronaviruses and toroviruses and the proteins encoded by ORF6 of LDV and EAV (Meulenberg *et al.*, 1993a). This suggests a similar membrane topology and membrane-associated function. In contrast to the amino acid sequence of ORFs 2 to 6, the amino acid sequence of ORF7 is basic: it does not contain hydrophobic segments. Because the amino acid sequence of ORF7 closely resembles that of other viral nucleocapsid proteins, it is thought to be the nucleocapsid protein of LV.

In vitro transcription and translation of ORFs 2 to 7

ORFs 2 to 7 were cloned in plasmid vector pGEM-4Z, and transcripts from these ORFs were translated in nuclease-treated rabbit reticulocyte lysate. Figure 1 shows the translation products analyzed by SDS–PAGE. The apparent sizes of the products, translated in the absence of microsomal membranes were 26 kDa for ORF2, 27 kDa for ORF3, 19 kDa for ORF4, 20 kDa for ORF5, 18 kDa for ORF6, and 15 kDa for ORF7. Although the sizes of these proteins, determined by SDS–PAGE, do not necessarily correlate with true molecular weights, they are in fair agreement with the sizes that were estimated from the amino acid sequence (Table 1). Translation of the transcripts of ORFs 2 to 5 in the presence of microsomal membranes resulted in larger products, the major protein bands being 30, 45, 31, and 25 kDa, respectively (Fig. 1). The presence of microsomal membranes during translation did not affect the sizes of the gene products of ORFs 6 and 7. The expression of the ORF6 gene product was somewhat higher when microsomal membranes were added during *in vitro* translation. Maybe these membranes enhance translation or increase the stability of the membrane protein encoded by ORF6. The results indicate that the gene products of ORFs 2 to 5 were glycosylated during *in vitro* translation, whereas the gene products of ORFs 6 and 7 were not. The shift in size of the glycosylated gene products of ORFs 3 and 4 with 7 and 4 putative glycosylation sites, respectively (Table 1), was greater than the shift in size of the gene products of ORFs 2 and 5 with both 2 putative N-glycosylation sites (Table 1). The glycosylation of the gene products of ORFs 2 to 5 that were synthesized in the presence of microsomal membranes was further confirmed by digesting the products with PNGaseF, an enzyme which cleaves off *N*-glycans. This procedure reduced the size of the gene products of ORFs 2 to 5 to the size of the unglycosylated gene products (data not shown). For ORFs 2, 4, and 5 also a product of smaller size than the unglycosylated products was observed, when translated in the presence of microsomal membranes (Fig. 1), and increased in intensity after PNGase treatment (data not shown). These products may represent unglycosylated gene products from which the N-terminal signal sequence is cleaved off during membrane insertion. For ORF2 a second protein of smaller size than the unglycosylated protein was observed upon

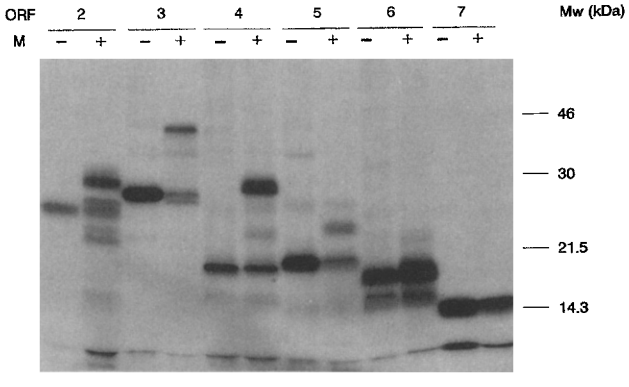

FIG. 1. Sodium dodecyl sulfate–polyacrylamide (15%) gel electrophoretic analysis of proteins encoded by ORFs 2 to 7 of Lelystad virus, under reducing conditions. The proteins were expressed by *in vitro* transcription and translation in the absence (–) or presence (+) of microsomal membranes (M) in the presence of L-[^{35}S]methionine. The L-[^{35}S]methionine-labeled proteins were analyzed by SDS–PAGE under reducing conditions and visualized by autoradiography. The position and size of marker proteins analyzed in the same gel are indicated at the right.

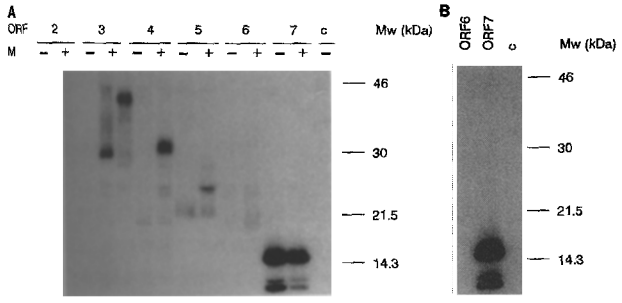

FIG. 2. RIP and SDS–PAGE of radiolabeled proteins encoded by ORFs 2 to 7 synthesized *in vitro*. The transcripts of the ORFs were translated in the absence (–) or presence (+) of microsomal membranes (M) and were immunoprecipitated with convalescent serum 21 collected from a pig infected with LV (A). As a control, the *in vitro* translation products of Brome mosaic virus (BMV) RNA (supplemented with the *in vitro* translation kit of Pharmacia) were immunoprecipitated with serum 21 (indicated as ORFC). The *in vitro* translation products of ORFs 6 and 7 and BMV RNA (indicated with C) were also immunoprecipitated with a monoclonal antibody SDOW17 raised against a United States isolate of LV (B). Identical precipitation reactions were obtained with monoclonal antibodies SDOW12, WBE1, and WBE4 to WBE6.

translation in the presence of microsomal membranes (Fig. 1). The nature of this protein is not understood. The translation products of ORFs 6 and 7, when generated in the presence of microsomal membranes, did not change in size after treatment with PNGaseF (data not shown). This finding indicates that the putative N-glycosylation sites of ORFs 6 and 7 are not in fact glycosylation sites, at least in *in vitro* experiments.

Immunoprecipitation of *in vitro* translation products of ORFs 2 to 7

The gene products of ORFs 2 to 7 synthesized in the presence or absence of microsomal membranes were immunoprecipitated with convalescent serum, serum 21, of a pig infected with LV. Serum 21 recognized the gene products of all these ORFs (Fig. 2A). The gene product of ORF7 was immunoprecipitated best, whereas the gene products of ORFs 2 and 6 were immunoprecipitated only weakly (and were observed clearly only after longer exposure; data not shown). The 12- and 13-kDa bands, which were coprecipitated with the major 15-kDa product of ORF7, are probably minor products formed as a result of proteolytic degradation, premature translational stop, or internal initiation of translation. Serum 21 did not immunoprecipitate *in vitro* translation products of Brome mosaic virus RNA, which served as negative control (Fig. 2A). These results indicate that the proteins encoded by ORFs 2 to 7 are made during virus replication in pigs and that antibodies are raised against all these proteins. Monoclonal antibodies raised against a United States isolate (SDOW12 and SDOW17; Nelson *et al.*, 1993) and an English isolate of LV (WBE1, WBE4 to WBE6; Drew *et al.*, 1994) all recognized the 15-kDa gene product of ORF7. Figure 2B shows a representative radio immunoprecipitation (RIP) assay using SDOW17.

Preparation of gene-specific antipeptide sera

Gene-specific antisera, containing antibodies directed against peptides of ORFs 2 to 7 were raised in rabbits. Five sera — 694 (anti-ORF3), 698 (anti-ORF4), 704 (anti-ORF5), 710 (anti-ORF6), and 714 (anti-ORF7) — were selected that reacted positively with the corresponding peptide in an ELISA and that reacted positively in an IPMA with alveolar macrophages infected with LV (Table 2). The latter finding indicates that these sera recognize LV-specific proteins. The antipeptide sera obtained for ORF2 reacted negatively in the IPMA. However, anti-ORF2 serum 690 immunoprecipitated the gene product of ORF2 synthesized *in vitro* in the presence of microsomal membranes, whereas the preserum did not recognize this product (Fig. 3; Table 2). Furthermore, no cross-reactivity of serum 690 with other *in vitro* translation products was observed, proving that the anti-ORF2 serum 690 is specific for the ORF2 product (data not shown). Similarly, antipeptide sera 694 (anti-ORF3), 698 (anti-ORF4), 704

TABLE 2

REACTIVITY OF SERA RAISED AGAINST LV-SPECIFIC PEPTIDES OF ORFs 2 TO 7, USING DIFFERENT TEST SYSTEMS

Serum	ORF	Amino acids[a]	Sequence	IPMA[b]	IVT[c]	WB[d]	RIP[e]
690	2	64–78	CTLPNYRRSYEGLLPN	–	+	–	–
694	3	75–92	CKIGHDRCEERDHDELLM	+	+	–	–
698	4	62–77	CQEKISFGKSSQCREAV	+	+	–	–
704	5	145–161	CNFIVDDRGRVHRWKSPI	+	+	+	–
710	6	154–171	CVLGGKRAVKRGVVNLVKY	+	–	+	–
714	7	43–60	CGGQAKKKKPEKPHFP	+	+	+	+

[a] Location of the peptide sequence in each ORF.
[b] Immunoperoxidase monolayer assay on macrophages infected with Lelystad virus.
[c] Immunoprecipitation of radiolabeled *in vitro* translation products of ORFs 2 to 7.
[d] Western blot analysis of purified Lelystad virus.
[e] Immunoprecipitation of radiolabeled cell lysates of LV-infected CL2621 cells.

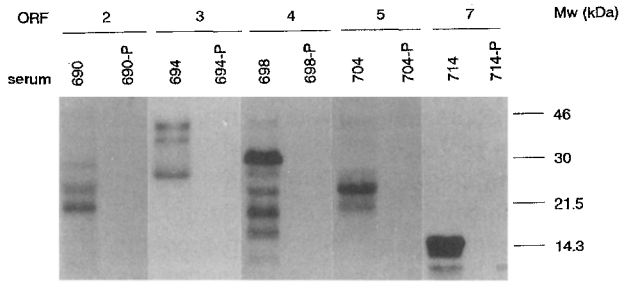

FIG. 3. RIP of *in vitro* translation products of ORFs 2 to 5 and 7, with gene-specific antipeptide sera raised in rabbits. The translation of transcripts was performed in the presence of microsomal membranes. The gene products of ORFs 2, 3, 4, 5, and 7 were immunoprecipitated with sera 690 (anti-ORF2), 694 (anti-ORF3), 698 (anti-ORF4), 704 (anti-ORF5), and 714 (anti-ORF7), respectively, and their corresponding preserum, which is indicated with the extension −P.

(anti-ORF5), and 714 (anti-ORF7) immunoprecipitated specifically the *in vitro* translation products of ORFs 3, 4, 5, and 7, respectively (Fig. 3; Table 2). The glycosylation of the *in vitro* translation products synthesized for these RIP experiments was less complete than that in previous experiments. Therefore, also partially glycosylated products were observed in the immunoprecipitation reactions with ORFs 2 to 5. Although serum 710 (anti-ORF6) reacted positively in the IPMA, it did not recognize the ORF6 gene product synthesized by *in vitro* translation in the RIP assay. We hypothesize that the conformation of ORF6 in this RIP assay is unfavorable for exposure of the linear peptide epitope recognized by serum 710.

Identification of structural proteins

The generated gene-specific antipeptide sera were used to identify the proteins incorporated in virus particles. Lelystad virus was purified on a glycerol-di-K-tartrate gradient or sucrose gradient, and infectious peak fractions, found at densities of 1.16–1.17 g/cm^3, were subjected to Western blot analyses using convalescent serum 21 and the gene-specific antipeptide sera. Serum 21 recognized three structural proteins with an apparent molecular weight (MW) of 25, 18, and 15 kDa (Fig. 4A). In addition to these three proteins, two faint bands of 28 and 42 kDa were observed. These were not detected on the control strip incubated with a negative pig serum. Antipeptide serum 704 (specific for ORF5), recognized the 25-kDa protein (E) and a faint band of 42 kDa. We concluded that the E protein is encoded by ORF5. The much fainter protein band observed at 42 kDa might be a homo- or heterodimer of the ORF5-encoded protein, still present to a limited extent under denaturing conditions. Antipeptide serum 710 stained an 18-kDa protein (M), indicating that this structural protein is expressed from ORF6. Antipeptide serum 714 reacted with a 15-kDa protein (major band) and a 28-kDa protein (minor band). This finding proves that the 15-kDa protein (N) is encoded by ORF7. The 28-kDa protein is probably a dimeric form of the N protein. No staining was observed when these Western blot strips were incubated with 704, 710, or 714 presera or with antipeptide sera specific for ORFs 2, 3, and 4.

Glycosidase treatment of purified LV

The results of the *in vitro* translation experiments indicated that the gene product of ORF5 was N-glycosylated, whereas the gene products of ORFs 6 and 7 were not. To establish whether these proteins are similarly glycosylated *in vivo,* we treated purified LV with PNGaseF.

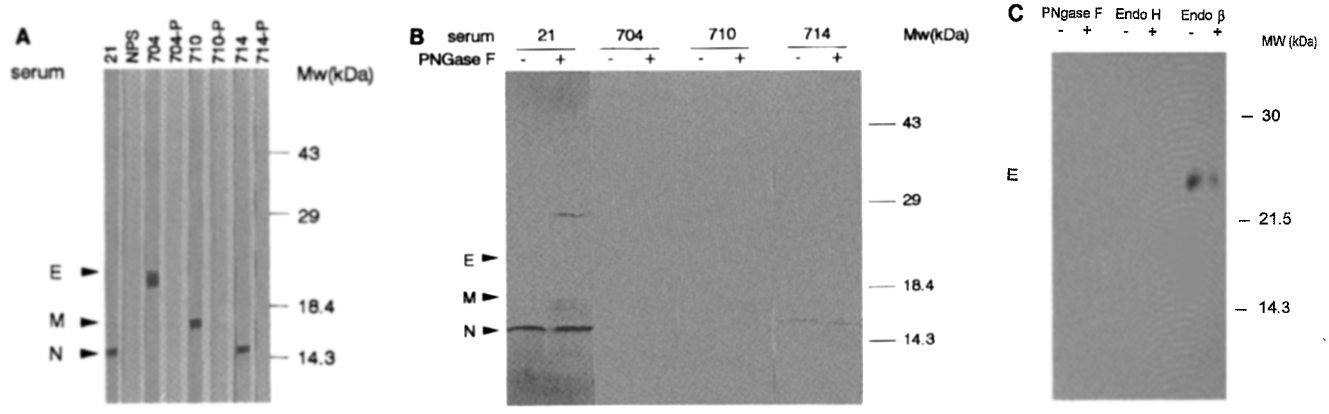

FIG. 4. Western blot analysis of virions of LV. Virions were purified by isopycnic sedimentation on a glycerol-di-K-tartrate or sucrose gradient. (A) Infectious fractions were resuspended in Laemmli sample buffer and were separated on a 12.5% polyacrylamide gel by SDS–PAGE. Proteins were transferred to nitrocellulose paper, and nitrocellulose strips were immunostained with porcine anti-LV serum 21, negative pig serum (NPS), gene-specific antipeptide sera 704, 710, and 714, and their corresponding presera, 704-P, 710-P, and 714-P. The positions of the 15-kDa N protein, the 18-kDa M protein, and the 25-kDa E protein are indicated with an arrowhead. (B) Samples of LV virions were treated with PNGaseF (+) or left untreated (−). After SDS–PAGE and electrotransfer to nitrocellulose paper, proteins were immunostained with porcine anti-LV serum 21 and gene-specific antipeptide sera 704, 710, and 714. The positions of the E, M, and N protein are indicated. (C) Samples of LV virions were treated with PNGaseF (+), EndoH (+), or endo-β-galactosidase (Endo-β, +) or left untreated (−). After SDS–PAGE and electrotransfer to nitrocellulose paper, the E protein was immunostained with antipeptide serum 704.

The treated and untreated virus preparations were analyzed on Western blots stained with convalescent serum 21 and the antipeptide sera specific for ORFs 5, 6, and 7. As is shown in Fig. 4B, after treatment with PNGaseF the apparent MW of the E protein was reduced to approximately 17 to 18 kDa. The apparent MW of the dimer of the E protein was reduced from 42 to 34 kDa. The size of the M and N proteins remained the same before and after treatment with PNGaseF. These results show that E is an N-glycosylated structural protein, whereas M and N are not. Furthermore, these results are in line with our endoglycosidase studies on *in vitro* translation products, mentioned above. If the apparent size difference (±7 kDa) between the unglycosylated and glycosylated E protein correlates with true molecular weight, this would suggest that both putative N-glycosylation sites are functional *in vivo*. The 17- to 18-kDa backbone of the unglycosylated E protein (which was also observed after PNGaseF treatment of the ORF5-encoded gene product translated in the presence of microsomal membranes) was smaller than the ORF5-encoded gene product (20 kDa) translated in the absence of microsomal membranes (Fig. 1). This finding suggests that the signal sequence of ORF5 is removed during membrane insertion. The E protein was also treated with EndoH (an endoglycosidase that digests N-linked high-mannose *N*-glycans but not complex *N*-glycans) and was shown to be resistant, although not completely (Fig. 4C). A shift in apparent molecular weight of ±1 kDa was observed. We cannot explain this slight reduction in size after EndoH treatment. The EndoH digestion of other substrates was complete, under identical conditions. Although proteins containing both EndoH-resistant and EndoH-sensitive oligosaccharides have been observed by others (Vennema *et al.*, 1990; Doyle *et al.*, 1986), a shift in apparent molecular weight of ±1 kDa, if correlating with true molecular weight, is too low to account for one EndoH-sensitive oligosaccharide. The E protein was completely resistant to endo-β-galactosidase (Fig. 4C). This indicates that E, in contrast to G_1, the counterpart of E in EAV (de Vries *et al.*, 1992), does not contain *N*-acetyllactosamine residues.

Immunoprecipitation of LV-specific proteins in cell lysates

In order to identify additional (non)structural LV-specific proteins, porcine anti-LV serum 21 and the gene-specific sera were also used to immunoprecipitate proteins from LV-infected L-[^{35}S]methionine- and L-[^{35}S]-cysteine-labeled cell lysates. Convalescent serum 21 was used to immunoprecipitate the E, M, and N proteins from LV-infected cell lysates (Fig. 5). The N protein was also immunoprecipitated with specific antipeptide serum for ORF7 (Table 2). The E, M, and N proteins could not be immunoprecipitated from mock-infected cells. Although the size of the M protein seemed to be somewhat larger in cell lysates (Fig. 5) than in the virion (Fig. 4), this size difference is most likely due to the use of a different percentage of acrylamide and a different gel electrophoresis system (mini-gel) for the gel of Fig. 5, compared to the gels prepared for the Western blots, shown in Fig. 4. The gene products of ORFs 2, 3, and 4 could not be detected with serum 21, nor with specific antipeptide sera, which were otherwise able to immunoprecipitate the *in vitro* translation products of ORFs 2 to 4.

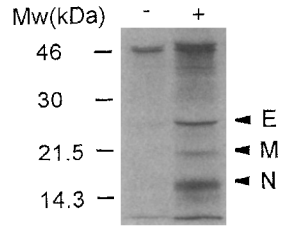

FIG. 5. Detection of LV-specific proteins in LV-infected CL2621 cells. CL2621 cells were infected at m.o.i. of 0.1 with LV or were mock-infected. At 24 hr after infection, cells were labeled with L-[^{35}S]-methionine and L-[^{35}S]cysteine for 16 hr. Cell lysates were tested in a RIP with porcine anti-LV serum 21. Immunoprecipitated proteins were analyzed by SDS–PAGE on a gel containing 15% polyacrylamide. The E, M, and N proteins are indicated.

DISCUSSION

Although the replication strategy of LV has been studied, and the complete nucleotide sequence of the viral genome has been determined, little is known about the structural proteins of LV. The purpose of our study was to analyze these proteins in more detail. Three protein components of LV virions were identified. These proteins have apparent molecular weights of 15, 18, and 25 kDa and are designated N, M, and E, respectively. The 15-kDa protein (N) is encoded by ORF7 and is not N-glycosylated. Its amino acid sequence is extremely basic and has 41 and 20% amino acid identity with the nucleocapsid protein of LDV (Chen *et al.*, 1993; Godeny *et al.*, 1990) and EAV (den Boon *et al.*, 1991a), respectively. The identity of the nucleocapsid proteins of EAV and LDV was established by virus fractionation experiments. After the virus particles were treated with detergent, only the N proteins of 15 to 16 kDa cosedimented with the viral genome in the bottom fractions of a sucrose gradient (Brinton-Darnell and Plagemann, 1975; Michaelides and Schlesinger, 1973; Zeegers *et al.*, 1976). In a sucrose gradient layered with NP-40-treated LV virions, the N protein was found in the bottom fraction of the gradient at a density of 1.18 g/cm^3, whereas the M and E proteins cosedimented in the middle of the sucrose gradient at a density of 1.10 g/cm^3. This supplies further evidence supporting the assumption that the 15-kDa N protein of LV is the nucleocapsid protein.

The 18-kDa protein (M) is a non-N-glycosylated envelope protein encoded by ORF6, which has the same hydrophobicity profile as the M proteins of MHV (Boursnell, 1984) and IBV (Rottier *et al.,* 1986), the E protein of BEV (den Boon *et al.,* 1991b), the M protein of EAV (de Vries *et al.,* 1992), and the gene product of ORF6 of LDV (Chen *et al.,* 1993; Godeny *et al.,* 1993). These proteins are characterized by the presence of three hydrophobic segments at the N-terminus. Protease protection experiments have shown that the M proteins of MHV-A59 and IBV are type III integral membrane proteins (Rottier *et al.,* 1984; Cavanagh *et al.,* 1986). They are anchored in the membrane by the three successive hydrophobic domains, whereas the C-terminal part is thought to be associated with the membrane surface. We assume that the M protein of LV has a similar membrane-associated topology.

The structural protein of 25 kDa, designated E, is the gene product of ORF5. We showed that E is N-glycosylated, probably at two different sites. The E protein incorporated in virus particles was sensitive to PNGaseF, but partially resistant to EndoH. These findings indicate that during virus maturation, E is transported through the Golgi apparatus and its N-linked oligosaccharides undergo Golgi-specific modifications. The apparent size of E decreased about 1 kDa when virions were treated with EndoH, indicating that it contains an EndoH-sensitive *N*-glycan moiety. However, if this reduction correlates with true molecular weight, it is too small to account for one *N*-glycan moiety being removed by EndoH. We cannot explain this reduction.

E is the counterpart of G_1, a structural envelope protein encoded by ORF5 of EAV (de Vries *et al.,* 1992). G_1 migrated as a heterogeneous protein of 30 to 42 kDa on SDS–PAGE, because a variable number of lactosamine repeats were added to the N-linked core oligosaccharide. The E protein of LV, however, was not susceptible to endo-β-galactosidase. Therefore the maturation of the N-linked oligosaccharide side chains of the E and G_1 protein is probably different. The topology of the E protein is still unknown. Both the E and G_1 proteins contain a large internal hydrophobic region, which might anchor these proteins in the membrane.

The N, M, and E proteins were detected both in Western blot analysis of purified virus and in RIP assays of cell lysates. These proteins were also detected by Nelson *et al.* (1993) in cell lysates of CL2621 cells infected with LV or with a United States isolate of LV. Although we have generated gene-specific antipeptide sera, which recognized the N-glycosylated *in vitro* translation products of ORFs 2, 3, and 4, we were not able to detect the proteins encoded by these ORFs in cell lysates or purified LV. Perhaps these proteins were expressed only at very low levels in CL2621 cells and only small amounts of these proteins were incorporated in virus particles. Furthermore, the affinity of the polyvalent sera and monospecific peptide sera might not be high enough to detect such low amounts of protein. Alternatively, the ORF2, 3, and 4 gene products may be present in heterocomplexes that are not denatured under the conditions used. We do not yet know whether the gene products of ORFs 2 to 4 are structural proteins. The hydrophobicity profile of ORF2 of LV is similar to that of ORF2 of EAV and LDV. The gene product of ORF2, a 25-kDa glycoprotein designated G_s, was detected in a purified virus preparation of EAV (de Vries *et al.,* 1992). It constituted only 1–2% of the virion protein and was not recognized by a polyvalent antivirion serum. Therefore it is not clear whether this protein is only incidentally incorporated into virions or is present in the virion preparation because it is contaminated with cellular proteins.

Although LV is able to grow in CL2621 cells, we were not able to perform a single step growth curve in these cells, since not all cells are infected after 1 hr absorption at an m.o.i. of 10. The virus growth and cell labeling conditions must be improved in order to increase the amount of label incorporated in proteins of LV, which may allow us to perform pulse–chase experiments in labeled cells and RIP assays on purified labeled LV particles. In addition, the generation of more monospecific or monoclonal antibodies recognizing the gene products of ORFs 2 to 6 will be useful for further elucidation of the function and topology of these proteins. Until now, only monoclonal antibodies recognizing the N protein of LV have been isolated (Nelson *et al.,* 1993; Drew *et al.,* 1994).

ACKNOWLEDGMENTS

We thank J. Langeveld for the synthesis and analysis of the synthetic peptides and P. Rottier for fruitful discussions. We thank D. Benfield and T. Drew for the supply of monoclonal antibodies. Part of this work was supported by Boehringer-Ingelheim, Germany, and the Produktschap voor Vee en Vlees (PVV), The Netherlands.

REFERENCES

BAIROCH, A., and BOECKMANN, B. (1991). Prosite, a dictionary of sites and patterns in proteins. *Nucleic Acids Res.* **19,** 2241–2245.

BOURSNELL, M. E. G., BROWN, T. D. K., and BINNS, M. M. (1984). Sequence of the membrane protein gene from avian coronavirus IBV. *Virus Res.* **1,** 303–314.

BRINTON-DARNELL, M., and PLAGEMANN, P. G. W. (1975). Structure and chemical–physical characteristics of lactate-dehydrogenase-elevating virus and its RNA. *J. Virol.* **16,** 420–433.

CAVANAGH, D., DAVIS, P. J., and PAPPIN, D. J. C. (1986). Coronavirus IBV glycopeptides: Locational studies using proteases and saponin, a membrane permeabalizer. *Virus Res.* **4,** 145–156.

CHEN, Z., KUO, L., ROWLAND, R. R. R., EVEN, C., FAABERG, K. S., and PLAGEMANN, P. G. W. (1993). Sequences of 3′ end of genome and of 5′ end of open reading frame 1a of Lactate dehydrogenase-elevating virus and common junction motifs between 5′ leader and bodies of seven sugenomic mRNAs. *J. Gen. Virol.* **74,** 643–660.

COLLINS, J. E., BENFIELD, D. A., CHRISTIANSON, W. T., HARRIS, L., HENNINGS, J. C., SHAW, D. P., GOYAL, S. M., MCCULLOUGH, S., MORRISON, R. B., JOO, H. S., GORCYCA, D. E., and CHLADEK, D. W. (1992). Isolation of swine infertility and respiratory syndrome virus (Isolate ATCC-VR-

2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.* **4,** 117–126.

CONZELMANN, K. K., VISSER, N., VAN WOENSEL, P., and TIEL, H. J. (1993). Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. *Virology* **193,** 329–339.

DEN BOON, J. A., SNIJDER, E. J., CHIRNSIDE, E. D., DE VRIES, A. A. F., HORZINEK, M. C., and SPAAN, W. J. M. (1991a). Equine arteritis virus is not a togavirus but belongs to the coronavirus superfamily. *J. Virol.* **65,** 2910–2920.

DEN BOON, J. A., SNIJDER, E. J., LOCKER, J. K., HORZINEK, M. C., and ROTTIER, P. J. M. (1991b). Another triple-spanning envelope protein among intracellularly budding RNA viruses: The torovirus E protein. *Virology* **182,** 655–663.

DE VRIES, A. A. F., CHINSIDE, E. D., HOZINEK, M. C., and ROTTIER, P. J. M. (1992). Structural proteins of equine arteritis virus. *J. Virol.* **66,** 6294–6303.

DOYLE, C., SAMBROOK, J., and GETHING, M.-J. (1986). Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. *J. Cell Biol.* **103,** 1193–1204.

DREW, T. W., SANDS, J. J., PATON, D. J., and MEULENBERG, J. J. M. (1994). Production, characterisation and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome (PRRSV). Manuscript in preparation.

GODENY, E. K., SPEICHER, D. W., and BRINTON, M. A. (1990). Map location of lactate dehydrogenase-elevating virus (LDV) capsid protein (Vp1) gene. *Virology* **177,** 768–771.

GODENY, E. K., CHEN, L., KUMAR, S. N., METHVEN, S. L., KOONIN, E. V., and BRINTON, M. A. (1993). Complete genomic sequence and phylogenetic analysis of the lactate-dehydrogenase-elevating virus (LDV). *Virology* **194,** 585–596.

HARTY, J. T., and PLAGEMANN, P. G. W. (1988). Formalin inactivation of the lactate dehydrogenase-elevating virus reveals a major neutralizing epitope not recognized during natural infection. *J. Virol.* **62,** 3210–3216.

HOPP, T. P., and WOODS, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78,** 3824–3828.

HULST, M. M., WESTRA, D. F., WENSVOORT, G., and MOORMANN, R. J. M. (1993). Glycoprotein E1 of Hog Cholera virus expressed in insect cells protects swine from hog cholera. *J. Virol.* **67,** 5435–5442.

JANIN, J. (1979). Surface and inside volumes of globular proteins. *Nature* **277,** 491–492.

KYTE, J., and DOOLITTLE, R. F. (1982). A simple method for displaying the hydrophatic character of a protein. *J. Mol. Biol.* **157,** 105–132.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

LOULA, T. (1990). Clinical presentation of mystery pig disease in the breeding herd and suckling piglets. *In* "Proceedings, Mystery Swine Disease Committee Meeting" October 6, 1990, Denver, Colorado. 29-31 Livestock Conservation Institute, Madison, WI.

MEULENBERG, J. J. M., HULST, M. M., DE MEIJER, E. J., MOONEN, P. L. J. M., DEN BESTEN, A., DE KLUYVER, E. P., WENSVOORT, G., and MOORMANN, R. J. M. (1993a). Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS) is related to LDV and EAV. *Virology* **192,** 62–74.

MEULENBERG, J. J. M., DE MEIJER, and MOORMANN, R. J. M. (1993b). Subgenomic RNAs of Lelystad virus contain a conserved junction sequence. *J. Gen. Virol.* **74,** 1697–1701.

MICHAELIDES, M. C., and SCHLESINGER, S. (1973). Structural proteins of lactic dehydrogenase-elevating virus. *Virology* **55,** 211–217.

MURTAUGH, M. P., ELAM, M., and KAKACH, L. (1993). Comparison of the structural protein coding sequences of Lelystad virus and VR2332 strains of the PRRS virus. *IXth Int. Congr. Virol. Abstracts,* 132.

NELSON, E. A., CHRISOPHER-HENNINGS, J., DREW, T., WENSVOORT, G., COLLINS, J. E., and BENFIELD, D. A. (1993). Differentiation of United states and european isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. *J. Clin. Microbiol.* **31,** 3184–3189.

PARKER, J. M., GUO, D., and HODGES, R. S. (1986). New hydrophilicity scale derived from high performance liquid chromatography peptide retention data: Correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry* **25,** 5425–5432.

PLAGEMANN, P. G. W., and MOENNIG, V. (1991). Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: A new group of positive-strand RNA viruses. *Adv. Virus Res.* **41,** 99–192.

PURCHIO, A. F., LARSON, R., and COLLET, M. S. (1984). Characterization of bovine viral diarrhea proteins. *J. Virol.* **50,** 666–669.

ROTTIER, P., BRANDENBURG, D., ARMSTRONG, J., VAN DER ZEIJST, B. A. M., and WARREN, G. (1984). Assembly in vitro of a spanning membrane protein of the endoplasmic reticulum: The E1 glycoprotein of coronavirus mouse hepatitis virus A59. *Proc. Natl. Acad. Sci. USA* **81,** 1421–1425.

ROTTIER, P. J. M., WELLING, G. W., WELLING-WESTER, S., NIESTERS, H. G. M., LENSTRA, J. A., and VAN DER ZEIJST, B. A. M. (1986). Predicted membrane topology of the coronavirus protein E1. *Biochemistry* **25,** 1335–1339.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SUTER, M. A. (1982). Modified ELISA techniques for anti-hapten antibodies. *J. Immunol. Methods* **53,** 103–108.

TOWBIN, H., STAEHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro cellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

VENNEMA, H., HEIJNEN, L., ZIJDERVELD, 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.

VAN BERLO, M. F., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1982). Equine arteritis virus-infected cells contain six polyadenylated virus-specific RNAs. *Virology* **118,** 345–352.

VON HEIJNE, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14,** 4683–4690.

WENSVOORT, G. (1993). Lelystad virus and the porcine epidemic aborion and respiratory syndrome. *Vet. Res.* **24,** 117–124.

WENSVOORT, G., DE KLUYVER, E. P., LUIJTZE, E. A., DEN BESTEN, A., HARRIS, L., COLLINS, J. E., CHRISTIANSON, W. T., and CHLADEK, D. (1992). Antigenic comparison of Lelystad virus and swine infertility and respiratory symdrome (SIRS) virus. *J. Vet. Diagn. Invest.* **4,** 134–138.

WENSVOORT, G., TERPSTRA, C., POL, J. M. A., TER LAAK, E. A., BLOEMRAAD, M., DE KLUYVER, E. P., KRAGTEN, C., VAN BUITEN, L., DEN BESTEN, A., WAGENAAR, F., BROEKHUIJSEN, J. M., MOONEN, P. L. J. M., ZETSTRA, T., DE BOER, E. A., TIBBEN, H. J., DE JONG, M. F., VAN'T VELD, P., GROENLAND, G. J. R., VAN GENNEP, J. A., VOETS, M. TH., VERHEIJDEN, J. H. M., and BRAAMSKAMP, J. (1991). Mystery swine disease in the Netherlands: The isolation of Lelystad virus. *Vet. Q.* **13,** 121–130.

ZEEGERS, J. J. W., VAN DER ZEIJST, and HORZINEK, M. C. (1973). The structural proteins of equine arteritis virus. *Virology* **73,** 200–205.