JOURNAL OF VIROLOGY, Oct. 1994, p. 6794–6798
0022-538X/94/$04.00+0

Vol. 68, No. 10

Recombinant Rabbit Hemorrhagic Disease Virus Capsid Protein Expressed in Baculovirus Self-Assembles into Viruslike Particles and Induces Protection

SYLVIE LAURENT,[1] JEAN-FRANÇOIS VAUTHEROT,[1] MARIE-FRANÇOISE MADELAINE,[1] GHISLAINE LE GALL,[2] AND DENIS RASSCHAERT[1]*

Unité de Virologie et d'Immunologie Moléculaires, Institut National de la Recherche Agronomique, 78350 Jouy en Josas,[1] and Centre National d'Etudes Vétérinaires et Alimentaires, 22440 Ploufragan,[2] France

Received 6 April 1994/Accepted 15 July 1994

VP60, the unique component of rabbit hemorrhagic disease virus capsid, was expressed in the baculovirus system. The recombinant VP60, released in the supernatant of infected insect cells, assembled without the need of any other viral component to form viruslike particles (VLPs), structurally and immunologically indistinguishable from the rabbit hemorrhagic disease virion. Intramuscular vaccination of rabbits with the VLPs conferred complete protection in 15 days; this protection was found to be effective from the fifth day after VLP injection and was accompanied by a strong humoral response.

Rabbit hemorrhagic disease virus (RHDV), the causative agent of a highly contagious disease in wild and domestic rabbits, was first described in China in 1984 (12). The disease was reported in Korea a year after and spread throughout Europe during the years 1987 to 1989 (19). Infected rabbits usually died within 48 to 72 h of necrotizing hepatitis and hemorrhages, but, surprisingly, animals less than 2 months old remained unaffected (32). The disease is responsible for great economic losses in rabbit production, which has been rapidly developing since 1980 (19). Recently characterized as a member of the *Caliciviridae* family (21, 24), the virion is 40 nm in diameter and shows a typical structured surface of regularly arranged cup-shaped depressions (21, 29). The genome, a 7.5-kb single-stranded positive RNA, shows substantial base homology with other calicivirus genomes, but its organization differs, as only two open reading frames (ORFs) can be detected on the RHDV genome, whereas the other calicivirus genomes contain three ORFs (9, 17, 20, 25). Together with the full-length genomic RNA, a 2.4-kb subgenomic RNA is found in the virion (17, 18, 21). In RHDV, ORF1 extends from nucleotide 10 to nucleotide 7042, coding for a polyprotein that is cleaved into nonstructural proteins and the capsid protein VP60, which in the other caliciviruses is encoded by a distinct ORF (17, 25). The N-terminal part of the polyprotein was found to contain consensus motifs delineated for the active sites of 2C-like helicase, 3C-like protease, and 3D-like polymerase of picornaviruses. In RHDV, the second ORF overlaps the first one by 17 nucleotides and codes for a yet-uncharacterized 12-kDa protein (18, 24). The in vitro translation of the subgenomic RNA showed it could code for VP60, but no detectable expression of ORF2 protein was reported (4). It still remains unclear whether VP60 is encoded by the 2.4-kb subgenomic RNA or cleaved from the polyprotein (ORF1) during the viral cycle (4, 25). The viral capsid seems to result from the multimerization of 180 copies of a single VP60 protein (27), which therefore appears to be a good candidate for the elicitation of a protective response (24). Both active immunization with VP60 alone and passive immunization by injection of antisera containing anti-VP60 antibodies were reported to afford protection against a viral challenge (24). However, because of the lack of a cell culture system allowing viral replication, the antigen for vaccine preparation is still extracted from livers of infected animals (2). The production of recombinant VP60 would both obviate the need to handle infectious material for vaccine preparation and be of great interest for studying VP60 oligomerization.

Recently we have cloned and sequenced the full-length genome of an RHDV isolate collected from a wild rabbit in France (25). The VP60 gene was cloned under the control of the baculovirus polyhedrin promoter. The recombinant protein self-assembled to form viruslike particles (VLPs) that could easily be purified from the supernatant of infected Sf9 cells. The ability of these particles to protect animals was also tested.

Production and purification of recombinant VP60 particles. Two different constructions of recombinant baculoviruses expressing the VP60 gene were made. Inserts G3 and G4 were obtained by PCR with pUG 5.151 (25) (Fig. 1) as the template and primers TCTAGAAGATCT**ATG**GAGGGCAAAGCCC GCAC, located at the putative start codon (bold) of the capsid gene, and TCTAGAAGATCTTTTATAAGCTTACTTTAAA CT, located at the 3′ end of the genome, for G3 and TCTAG AAGATCT**ATG**GAGGCAGCAGGCACTGCCAC, located 30 bp downstream from the start codon of the capsid gene, at a Q/G potential cleavage site of the 3C-like protease, with an in-frame translation start codon (bold) and TCTAGAAG ATCTAGTCCGATGAATT**CA**GACAT, located at the stop codon (bold) of the capsid gene, for G4. A *Bgl*II cleavage site (underlined) was added in each primer. The amplification products, digested by *Bgl*II, were ligated into the transfer vector pVL941 (14, 16) at the *Bam*HI- and *Bgl*II-compatible restriction site, to produce pVLG3 and pVLG4. Wild-type baculovirus AcRP6-SC DNA, previously linearized with *Bsu*36I restriction enzyme (10), was cotransfected, with either pVLG3 or pVLG4, in Sf9 cells by using lipofectin (GIBCO-BRL) (22). Positive recombinant baculoviruses Bac G3.12 and Bac G4.18 were identified by hybridization of dot-blotted DNA or by indirect immunofluorescence staining with monoclonal

* Corresponding author. Mailing address: Unité de Virologie et d'Immunologie Moléculaires, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78350 Jouy en Josas, France. Phone: 33 1 34 65 26 15. Fax: 33 1 34 65 26 21.

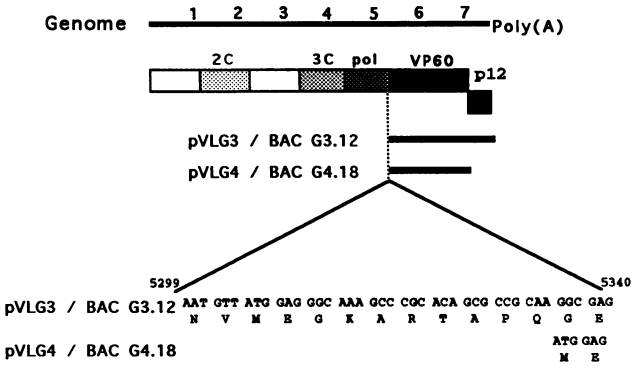

FIG. 1. Localization of the two different fragments of the RHDV genome cloned into the baculovirus transfer vector. The upper bar represents the calicivirus RNA genome, and underneath is represented the genomic organization with the putative polypeptides (17). PCR with the pUG 5.151 clone as a template and ad hoc oligonucleotides provided two inserts, which were cloned into pVL941 to provide pVLG3 and pVLG4. The two plasmids were used to produce Bac G3.12 and Bac G4.18 recombinant baculoviruses.

antibody (MAb) E3. This anti-VP60 MAb was selected from a library of MAbs resulting from the fusion of Sp2/0-Ag14 myeloma cells with lymph node lymphocytes of BALB/c mice immunized with purified RHDV (29a). Recombinant baculoviruses were plaque purified twice and used to prepare virus stocks.

The recombinant VP60 appeared to be released in the supernatant of Bac G3.12- and Bac G4.18-infected cells, where it could be detected by a sandwich enzyme-linked immunosorbent assay (ELISA) (15). For this assay, the anti-RHDV polyclonal serum was used to coat the solid phase at a dilution of 1/1,000 and anti-VP60 MAb E3 was used as a probe at a dilution of 1/100. The same test was used to monitor the

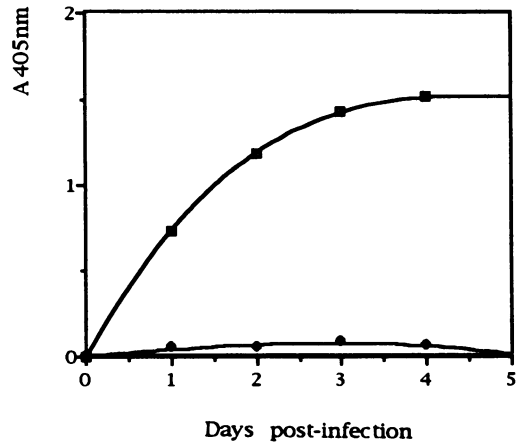

FIG. 2. Kinetics of release of the recombinant protein in the culture medium. A 96-well plate was infected with the recombinant baculovirus Bac G3.12 at a high multiplicity of infection, and the culture medium was harvested each day for a week. The presence of recombinant protein was detected by sandwich ELISA using an anti-RHDV polyclonal antiserum to capture the antigen and MAb E3 to detect the bound material. ◆, AcRP6-SC wild baculovirus; ■, Bac G3.12.

kinetics of recombinant VP60 production in the supernatant of infected cells (Fig. 2). The protein was present in the medium from the first day postinfection, and its production reached a maximum at the fourth day postinfection. VLPs were detected by electron microscopy after negative staining with 2% uranyl acetate in the supernatant of cells infected by both recombinant baculoviruses. These particles were purified from culture medium harvested 96 h postinfection, clarified, and then ultracentrifuged in a Beckman 45-Ti rotor at 40,000 rpm for 2 h. The pelleted material was suspended in a solution of CsCl (0.45 g/ml) before being centrifuged in a Beckman TL100.3 rotor at 100,000 rpm for 2 h. A single opalescent band could be visualized under indirect light against a dark background. The band was collected, diluted five times in water, and ultracentrifuged under the same conditions as used before. The pellet was finally resuspended in water and stored at 4°C. The yield of recombinant VP60 was estimated to be 20 mg/10^9 cells. VLPs, which were similar in size and appearance to the authentic RHDV, could be observed by electron microscopy (Fig. 3). The majority of these particles appeared to be electron dense (Fig. 3B). However, the lack of a major form of nucleic acid was deduced both from the buoyant density measurements, which were 1.31 g/cm^3 for the VLPs versus 1.36 g/cm^3 for the virus, and from the A_{260}/A_{280} ratio of the VLPs, which was found to be 1.1 instead of 1.44 for the virus preparation (24). These particles, like RHDV, were able to agglutinate the human group O erythrocytes (5a).

The results in this paper address the question of whether VP60 alone assembles to form the capsid or whether it needs expression of the ORF2 gene. The latter is present in the G3.12 construction, partially overlapping the 3′ end of the capsid gene. Herbert and Brown (7) have proposed a new termination-reinitiation mechanism for the translation of the third ORF of feline calicivirus (FCV), which could occur within the coupled ORF2 stop codon-ORF3 start codon region. This mechanism would account for the translation of the short ORF present at the 3′ end of all calicivirus genomes without the need for the synthesis of a specific mRNA. However, for Norwalk virus as well as for RHDV, no detectable expression of the low-M_r protein can be observed in recombinant baculovirus-infected cells (8). Nevertheless, we could not exclude that the ORF2 protein was synthesized at a low level and played a role in viral particle assembly. The VLPs produced with Bac G4.18 were, under electron microscopic examination, indistinguishable from those obtained with Bac G3.12 (data not shown), and this finding confirmed that the capsid protein was able to self-assemble in the absence of any other viral component.

Antigenicity of the recombinant protein. Antigenicities of the VLP and purified RHDV were compared by Western blot (immunoblot) analysis (1, 11). The anti-RHDV polyclonal serum was used at a 1/1,000 dilution, and MAb E3 was used at a 1/100 dilution. The polyclonal serum (Fig. 4) and MAb E3 (data not shown) recognized the same proteins both in RHDV and in VLP samples. The 60,000-molecular-weight protein (60K protein) corresponding to the monomer form of the capsid protein was immunodetected in both samples (Fig. 4A). In the VLP sample, a minor band migrating close to the recombinant VP60 was also consistently detected (Fig. 4). A similar result has been described for the core protein of hepatitis B virus (HBV) expressed in the baculovirus system (3). In the latter case, the minor band was found to be a polyhedrin-HBV core fusion protein which was initiated at the AUU mutated polyhedrin gene start codon of pVL941 (3, 14). The AUG of the RHDV capsid gene is also in frame, 35 bp downstream, with the AUU modified codon, thus allowing

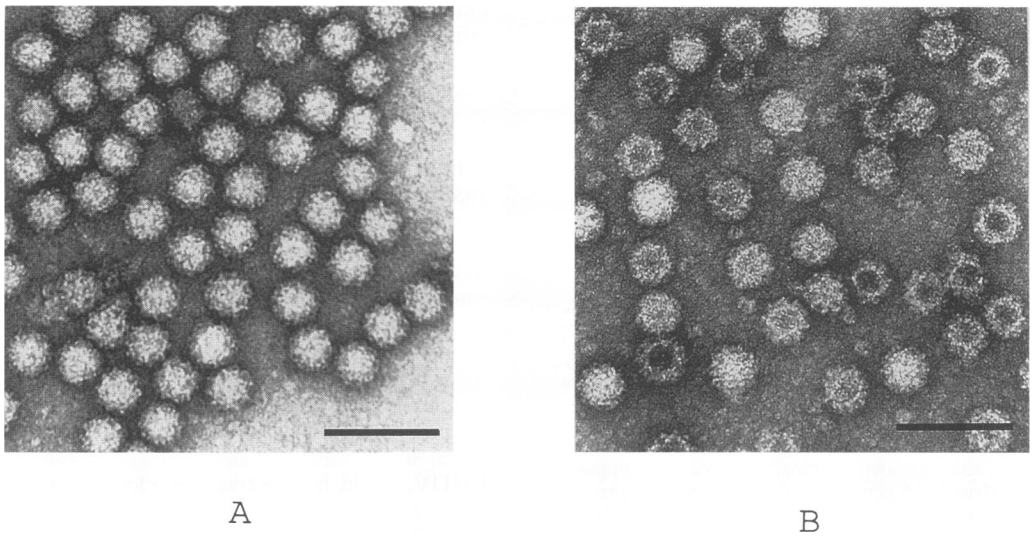

FIG. 3. Electron micrographs of RHDV from infected rabbit liver (A) and VLPs from supernatant of Sf9 cells infected with Bac G3.12 (B), both purified by CsCl gradient. Particles were adsorbed onto carbon-coated grids, stained with uranyl acetate (2%), and examined immediately. Bar, 100 nm.

expression of a fusion protein with an M_r of 62,000. Under reducing conditions, two proteins of 36 and 26 kDa were also revealed in the RHDV sample, whereas a 40-kDa product was detected in the VLP sample (Fig. 4). The 36- and 26-kDa proteins have already been described for an RHDV purified sample (17, 26), and proteins with similar M_rs, 40,000 for FCV (5) and 30,000 for Norwalk virus (6), were observed in other calicivirus preparations. Whether the 40-kDa protein immunodetected in the VLP sample, by polyclonal antibodies and MAbs, is a degradation or a cleavage product of VP60 is still under investigation (Fig. 4A).

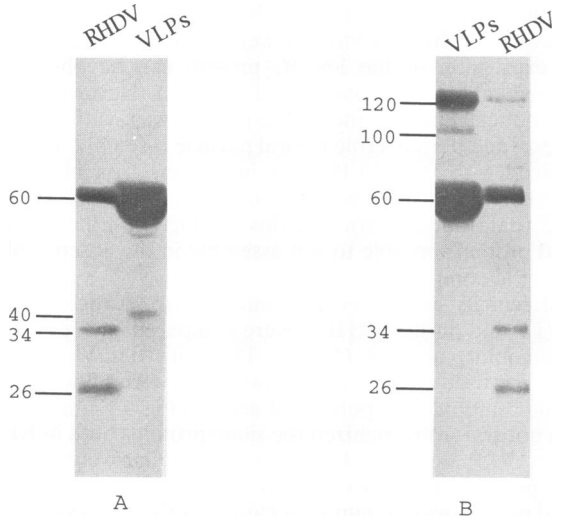

FIG. 4. Western blot analysis of the VP60 recombinant particles. VLPs present in the supernatant of Bac G3.12-infected cells were purified in a self-forming CsCl gradient. Recombinant VP60 protein and purified RHDV were solubilized at 100°C, either in the presence (A) or in the absence (B) of reducing agent (2-mercaptoethanol), electrophoresed on a 0.1% sodium dodecyl sulfate–10% polyacrylamide gel, and blotted onto a nitrocellulose membrane. Viral proteins were detected with an anti-RHDV polyclonal antiserum and revealed with an anti-rabbit immunoglobulin G alkaline phosphatase conjugate. Numbers indicate molecular weight (in thousands).

A 120-kDa protein, a possible dimer of VP60, was revealed in RHDV and VLP samples when they were boiled and dissociated under nonreducing conditions (Fig. 4B). Analysis of two VP60 amino acid sequences shows the existence of only one cysteine conserved at position 274, through which a disulfide bond maintaining the dimer could occur (17, 25). Furthermore, under nonreducing conditions, a 100-kDa protein, which could result from the association of the 60-kDa and the 40-kDa proteins by disulfide bond if the latter is a cleavage product of VP60 containing the cysteine residue, was revealed only in the VLP sample.

Vaccination of rabbits with the recombinant VP60. To investigate the immunogenicity of the recombinant protein, two groups (G1 and G2) of 10 specific-pathogen-free rabbits were vaccinated with 100 μg of purified VLPs produced with Bac G3.12. The VLPs were emulsified in Freund's complete adjuvant and injected intramuscularly either 15 (G1) or 5 (G2) days before a challenge of 1,000 50% lethal doses. All rabbits that were vaccinated 15 days before challenge survived and progressively developed high anti-VP60 antibody titers as measured in a direct ELISA. In this assay, the viral antigen (10 μg/ml) was bound to the solid phase by incubation for 16 h at room temperature in PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)] buffer (50 mM, pH 6.4) containing 150 mM NaCl. The subsequent steps (blocking of unreacted sites, incubation with serum dilutions and anti-immunoglobulin G conjugates) were adapted from an ELISA developed in our laboratory (30). The mean titers increased from 300 at 10 days after vaccination to 3,000 on the day of challenge (Fig. 5). In the second group of rabbits (G2), which received the vaccine only 5 days before challenge, one rabbit died with clinical and anatomopathological signs of RHD 24 h after challenge. RHDV could be recovered from the liver of this animal, which, interestingly, was the only one of the 10 which had an antibody titer of <20. The nine other rabbits had anti-VP60 antibody titers ranging between 35 and 125 on the day of the challenge. No RHDV could be detected by ELISA in the livers collected from all vaccinated surviving animals, which were sacrificed 7

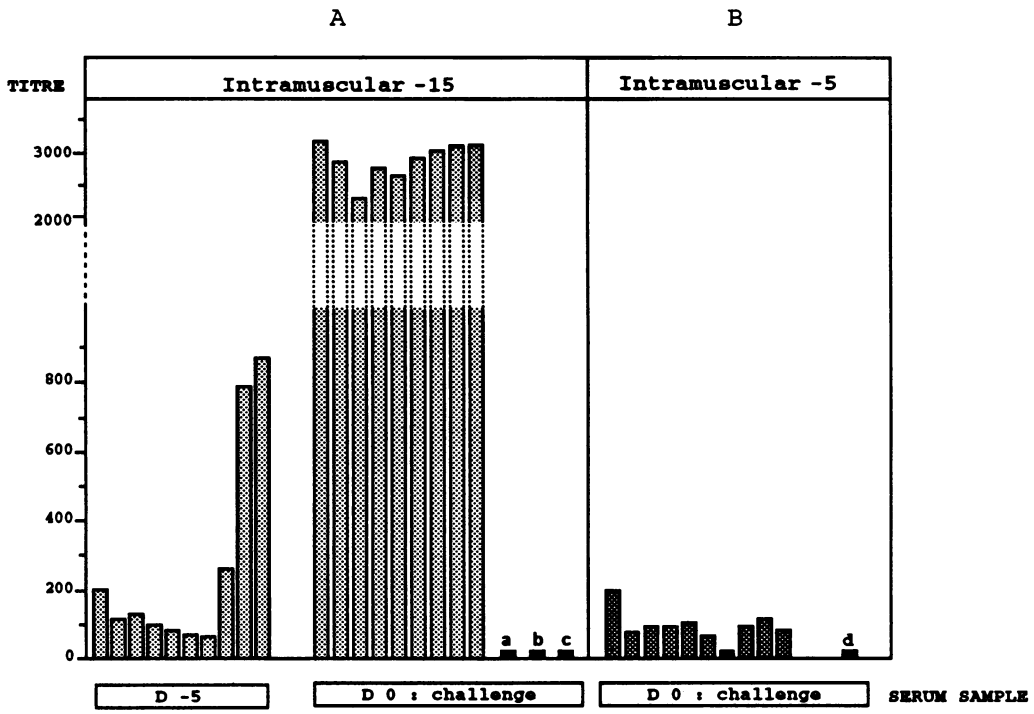

FIG. 5. Kinetics of antibody response in sera from VLP-vaccinated rabbits. Two groups of 10 rabbits were inoculated intramuscularly with VLPs emulsified in Freund's complete adjuvant (FCA) 15 days (A) or 5 days (B) before virulent challenge. Serum samples were collected 10 days after vaccination (A) and on the day of challenge (A and B); anti-RHDV antibodies were titrated in an ELISA for which purified RHDV was used to coat the plate. The bars labeled a, b, c, and d correspond to four control groups of five rabbits. Rabbits in group a were not inoculated, those in groups b and c were inoculated 15 days before challenge, with wild baculovirus and FCA, respectively (A), and animals in group d received FCA 5 days before challenge (B). Solid vertical bars correspond to the sera from dead rabbits with titers below 20.

days after challenge. In contrast, all animals belonging to the three control groups (five rabbits per group, four control groups) that received either wild baculovirus (15 days before challenge) or Freund's complete adjuvant alone (5 or 15 days before challenge) or were left untouched died between 24 and 48 h after challenge and had anti-VP60 titers of <20. RHDV could be detected in all liver homogenates from these animals.

Until now, the commercially available vaccines against RHDV have been produced from livers of infected rabbits. The baculovirus Sf9 cell expression system allows high-level production of recombinant proteins which have been proven to be potent antigens for vaccination purposes (13, 28, 31). Injection of VLPs was able to protect rabbits against a virulent RHDV challenge under the conditions used for vaccine testing in France. Furthermore, our findings that 9 of 10 rabbits vaccinated 5 days before challenge were protected against RHDV and that the one rabbit which did not survive died of the disease were consistent with the observations that anti-RHDV protective immunity is rapidly established in infected domestic-rabbit colonies (2). The anti-VP60 seroconversion, as measured by ELISA, was similar to the one observed when anti-RHDV antibodies were titrated in a hemagglutination inhibition assay, which revealed antibody production between the third and seventh days after vaccination with inactivated virus (2). In our experiments, anti-VP60 antibodies could be detected as early as 5 days after the vaccination and the titers progressively increased over a 15-day period. Because the once vaccinated animal which died of RHD did not develop any antibody response, we assume that the humoral response plays a key role in protection against the disease, as previously reported from antibody transfer experiments (24).

In addition to having outstanding immunogenicity, recombinant VLPs will be of great help in investigating capsid assembly and RNA-packaging processes and in identifying cell membrane structures acting as potential receptors for RHDV.

This work was supported in part by an Office National de la Chasse grant.

REFERENCES

1. **Aponte, C., N. M. Mattion, M. K. Estes, A. Charpilienne, and J. Cohen.** 1993. Expression of two bovine rotavirus non-structural proteins (NSP2, NSP3) in the baculovirus system and production of monoclonal antibodies directed against the expressed proteins. Arch. Virol. **133**:85–95.
2. **Argüello Villares, J. L.** 1991. Viral haemorrhagic disease of rabbits: vaccination and immune response. Rev. Sci. Tech. Off. Int. Epizoot. **10**:471–480.
3. **Beames, B., S. Braunagel, M. D. Summers, and R. E. Landford.** 1991. Polyhedrin initiator codon altered to AUU yields unexpected fusion protein from a baculovirus transfer vector. BioTechniques **11**:378–383.
4. **Boga, J. A., M. S. Marin, R. Casais, M. Prieto, and F. Parra.** 1992. In vitro translation of a subgenomic mRNA from purified virions of the Spanish field isolate AST/89 of rabbit hemorrhagic disease virus (RHDV). Virus Res. **26**:33–40.
5. **Carter, M. J., E. G. Routledge, and G. L. Toms.** 1989. Monoclonal antibodies to feline calicivirus. J. Gen. Virol. **70**:2197–2200.

5a. **Gagnière, J. P.** Personal communication.

6. **Greenberg, H. B., J. R. Valdesuso, A. R. Kalica, R. G. Wyatt, V. J. McAuliffe, A. Z. Kapikian, and R. M. Chanock.** 1981. Proteins of Norwalk virus. J. Virol. **37**:994–999.
7. **Herbert, T. P., and T. D. K. Brown.** 1993. Translational control of ORF3 in feline calicivirus, abstr. W7-2, p. 25. Abstr. IXth Int. Congr. Virol. 1993.

8. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of Norwalk virus capsid protein. J. Virol. **66**:6527–6532.
9. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195**:51–61.
10. **Kitts, P. A., M. D. Ayres, and R. D. Possee.** 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. **18**:5667–5672.
11. **Labbé, M., A. Charpilienne, S. E. Crawford, M. K. Estes, and J. Cohen.** 1991. Expression of rotavirus VP2 produces empty core-like particles. J. Virol. **65**:2946–2952.
12. **Liu, S. J., H. P. Xue, B. Q. Pu, and N. H. Qian.** 1984. A new viral disease in rabbits. Anim. Husb. Vet. Med. **16**:253–255.
13. **López de Turiso, J. A., E. Cortés, C. Martínez, R. Ruiz de Ybáñez I. Simarro, C. Vela, and I. Casal.** 1992. Recombinant vaccine for canine parvovirus in dogs. J. Virol. **66**:2748–2753.
14. **Luckow, V. A., and M. D. Summers.** 1988. High level expression of non fused foreign genes with polyhedrosis virus expression vectors. Virology **170**:31–39.
15. **MacCullough, K. C., and R. Butcher.** 1982. Monoclonal antibodies against foot-and-mouth disease virus 146S and 12S particles. Arch. Virol. **74**:1–9.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Meyers, G., C. Wirblich, and H. J. Thiel.** 1991. Rabbit hemorrhagic disease virus-molecular cloning and nucleotide sequencing of a calicivirus genome. Virology **184**:664–676.
18. **Meyers, G., C. Wirblich, and H. J. Thiel.** 1991. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. Virology **184**:677–686.
19. **Morisse, J. P., G. Le Gall, and E. Boilletot.** 1991. Hepatitis of viral origin in Lepidorae: introduction and aetiological hypotheses. Rev. Sci. Tech. Off. Int. Epizoot. **10**:283–295.
20. **Neill, J. D.** 1992. Nucleotide sequence of the capsid protein gene of two serotypes of sea lion virus: identification of conserved and non conserved amino acid sequences among calicivirus capsid proteins. Virus Res. **24**:211–222.
21. **Ohlinger, V. F., B. Haas, G. Meyers, F. Weiland, and H. J. Thiel.** 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. J. Virol. **64**:3331–3336.
22. **O'Reilly, D. R., L. K. Miller, and V. A. Luckow.** 1992. Baculovirus expression vectors. W. H. Freeman and Co., New York.
23. **Parra, F., J. A. Boga, M. S. Marin, and R. Casais.** 1993. The amino terminal sequence of VP60 from hemorrhagic disease virus supports its putative subgenomic origin. Virus Res. **27**:219–228.
24. **Parra, F., and M. Prieto.** 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. **64**:4013–4015.
25. **Rasschaert, D., S. Huguet, M. F. Madelaine, and J. F. Vautherot.** Sequence and genomic organization of a rabbit haemorrhagic disease virus isolated from a wild rabbit. Virus Genes, in press.
26. **Rodak, L., M. Granatova, L. Valicek, B. Smid, T. Vesely, and Z. Nevorankova.** 1990. Monoclonal antibodies to rabbit haemorrhagic disease virus and their use in the diagnosis of infection. J. Gen. Virol. **71**:2593–2598.
27. **Schaffer, F. L., and M. E. Soergel.** 1976. Single major polypeptide of a calicivirus: characterization by polyacrylamide gel electrophoresis and stabilization of virion by crosslinking with dimethyl suberimidate. J. Virol. **19**:925–931.
28. **Vakharia, V. N., D. B. Snyder, J. He, G. H. Edward, P. K. Savage, and S. A. Mengel-Wherat.** 1993. Infectious bursal disease virus structural proteins expressed in a baculovirus recombinant confer protection in chickens. J. Gen. Virol. **74**:1201–1206.
29. **Valicek, L., B. Smid, and J. Kudrna.** 1990. Electron and immunoelectron microscopy of rabbit haemorrhagic disease virus (RHDV). Arch. Virol. **112**:271–275.
29a. **Vautherot, J. F.** Unpublished data.
30. **Vautherot, J. F., M. F. Madelaine, P. Boireau, and J. Laporte.** 1992. Bovine coronavirus peplomer glycoproteins: detailed analyses of S1, S2 and HE. J. Gen. Virol. **73**:1725–1737.
31. **Vlak, J. M., and R. J. A. Keus.** 1990. Baculovirus expression vector system for production of viral vaccines, p. 91–128. *In* A. Mizrahi (ed.), Viral vaccines. Wiley-Liss, Inc., New York.
32. **Xu, Z. J., and W. X. Chen.** 1989. Viral haemorrhagic disease in rabbits: a review. Vet. Res. Commun. **13**:205–212.