JOURNAL OF VIROLOGY, May 1991, p. 2761–2765
0022-538X/91/052761-05$02.00/0

Vol. 65, No. 5

Live Attenuated Pseudorabies Virus Expressing Envelope Glycoprotein E1 of Hog Cholera Virus Protects Swine against both Pseudorabies and Hog Cholera

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Received 3 October 1990/Accepted 7 February 1991

To investigate whether live attenuated pseudorabies virus (PRV) can be used as a vaccine vector, PRV recombinants that expressed envelope glycoprotein E1 of hog cholera virus (HCV) were generated. Pigs inoculated with these recombinants developed high levels of neutralizing antibodies against PRV and HCV and were protected against both pseudorabies and hog cholera (classical swine fever).

Pseudorabies virus (PRV), an alpha herpesvirus, induces the economically important neurological disease pseudorabies (Aujeszky's disease) in swine (1, 6). Live attenuated PRV vaccines are used successfully to control this disease, which is prevalent in most parts of the world. The genome of PRV is a linear DNA molecule of about 150 kbp (18) into which foreign genes can be stably inserted (8, 21, 31). This makes attenuated PRV a promising candidate for the development of a live vaccine vector, conferring protection against both pseudorabies and other swine diseases, such as hog cholera (classical swine fever). Hog cholera is a contagious disease, characterized by high fever, multiple hemorrhages, and death, and is caused by hog cholera virus (HCV) (22). HCV is a pestivirus, recently classified as belonging to the *Flaviviridae* (4). Three major structural proteins have been described for HCV, two envelope glycoproteins (E1 and E2) and a nonglycosylated capsid protein (30). Pigs can be protected against hog cholera by vaccination with attenuated HCV (20). It is not yet clear which antigens of HCV elicit a protective immune response. Protected animals invariably have antibodies against E1 (26), a protein with a relative molecular weight of 51,000 to 54,000 (27). Furthermore, strongly neutralizing monoclonal antibodies directed against E1 have been prepared (25, 27). The E1 gene was therefore chosen to be inserted into the PRV genome.

The HCV genome is a positive-stranded RNA molecule of 12.3 kb containing a single open reading frame of 3,898 amino acids (9, 11, 12). The genome is probably translated into one polyprotein, which is then processed to the mature viral proteins. The location of the region encoding E1 on the HCV genome has recently been mapped by using an antiserum raised against a synthetic peptide, the sequence of which was predicted from the nucleotide sequence (12). The N and C termini of the protein are not known. However, by analyzing potential proteolytic cleavage sites, the N terminus of mature E1 was predicted to be located between amino acids 689 and 690 of the polyprotein. Given the molecular mass of E1 and its presence in the viral membrane, the C terminus is probably located within a hydrophobic region between amino acids 1050 and 1300 (12). From a cDNA clone of HCV strain Brescia (29), we have isolated three E1-encoding fragments which differ at the 3′ end: a 1,020-bp *Nae*I-*Eco*RI fragment which encodes an E1 variant without a transmembrane domain, an 1,118-bp *Nae*I-*Acc*I fragment which encodes an E1 variant with one transmembrane domain, and a 1,376-bp *Nae*I-*Eco*RV fragment which encodes an E1 variant with three transmembrane domains. *Nae*I cleaves the cDNA clone between the first 2 nucleotides of the codon for amino acid residue 690.

Previously we constructed an effective live attenuated PRV vaccine (strain 783), by deleting parts of the genome of the virulent PRV strain NIA-3 (5, 12). Here we used PRV clones pHBDelta2.4 (16) and pMZ64, which each contain one of these deletions, to construct attenuated PRV recombinants expressing HCV E1. Plasmid pHBDelta2.4 contains the unique short region of PRV from which the gI gene and part of the 11K gene are deleted. In pHBDelta2.4, the 1,379-bp *Bal*I-*Nde*I fragment, harboring the major part of the coding region of the nonessential glycoprotein gX (17), was replaced by HCV fragments encoding E1 (Fig. 1A). The HCV fragments were fused in frame with the first 58 bp of the coding region of gX, which resulted in the generation of a GGG codon (coding for glycine) at the fusion site. The fusion protein is predicted to be cleaved by a signal peptidase between this glycine residue and the adjacent leucine residue, which is encoded by the HCV sequence (residue 691 of the polyprotein [12]). Translational stop codons were created by cloning specific oligonucleotide linkers immediately downstream of the 3′ ends of the HCV fragments. Recombinant viruses carrying the E1 gene were generated by in vivo overlap recombination as described previously (23). The inserts of the E1-containing plasmids pMZ69, pMZ68, and pMZ67 were cotransfected into PK-15 cells with four overlapping cloned subgenomic PRV fragments, one of which (pMZ64) contained a deletion in the thymidine kinase gene (Fig. 1B). This resulted in the attenuated recombinant viruses M203, M204, and M205, encoding E1 without or with one or three transmembrane domains, respectively. PRV mutant M206, containing the same PRV-specific deletions in its genome as the recombinant viruses but lacking E1, was constructed to be the control (Fig. 1). The integrity of the

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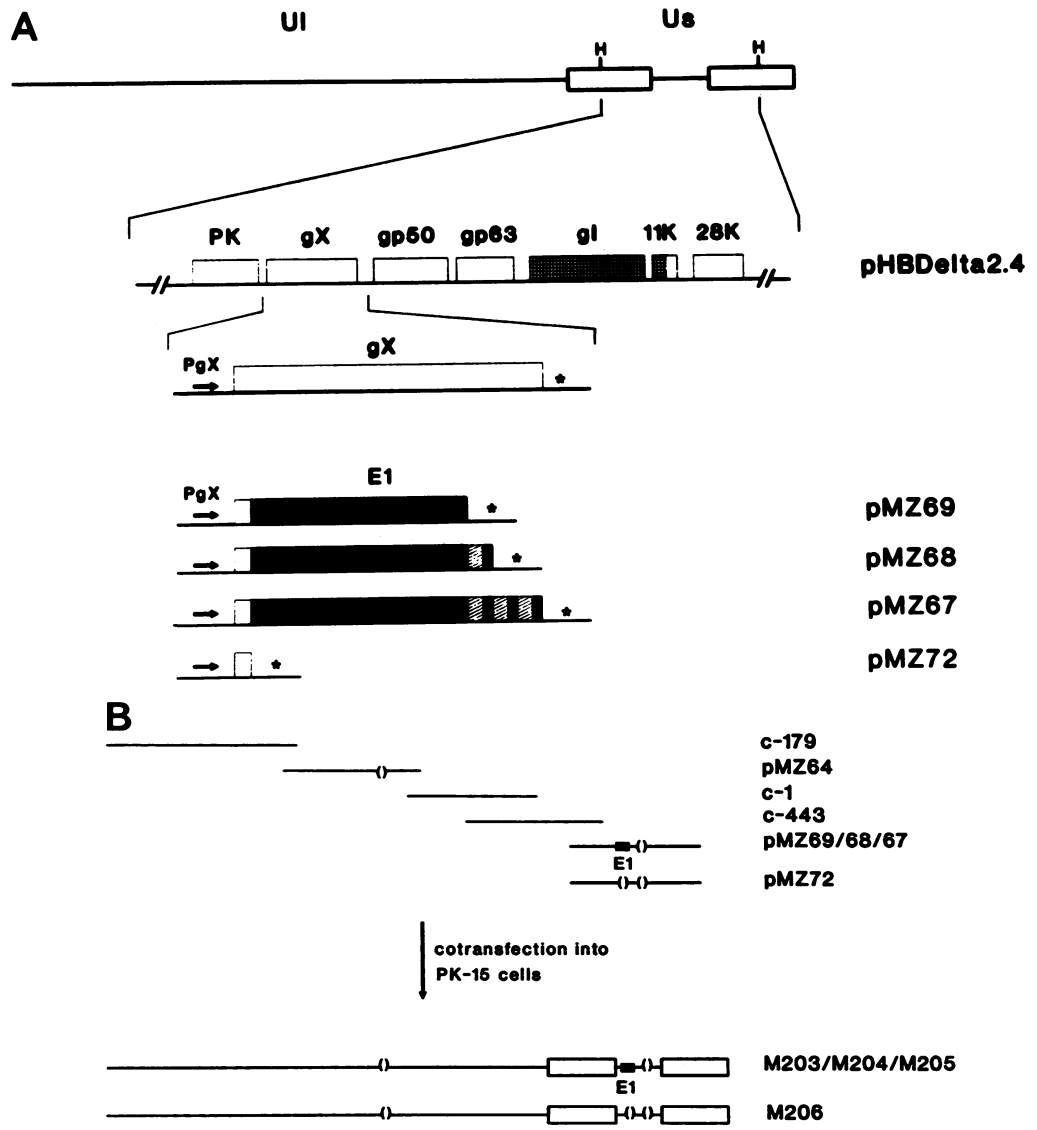

FIG. 1. Construction of recombinant PRV M203, M204, and M205, expressing HCV glycoprotein E1, and control virus M206. (A) Insertion of HCV sequences encoding E1 into a plasmid containing the unique short region (Us) of PRV (pHBDelta2.4). In the top panel a schematic representation of the PRV genome is shown. Open boxes represent inverted repeats. In the middle panel an expansion of the *Hin*dIII insert of pHBDelta2.4 is shown, with the genomic organization of the Us (13–15, 17, 24). The region of the Us deleted in this plasmid is marked by shading. The deletion comprises 2,055 bp and ranges from nucleotide sequence 5′-AGAAGCTGG. . ., located immediately downstream of the open reading frame encoding glycoprotein gp63, up to and including nucleotide sequence . . . .TACTACAGC-3′, located within the open reading frame encoding the 11,000-molecular-weight protein (11K protein). In the bottom panel the four constructs are shown that were made to generate the mutant viruses. The major part of the coding region of gX in pHBDelta2.4 was replaced by three HCV fragments (black boxes, including the hatched areas) differing at the 3′ end and encoding E1 without a transmembrane domain (pMZ69) or with one (pMZ68) or three (pMZ67) transmembrane domains. The hatched areas represent the sequences encoding the transmembrane domains. The HCV fragments were fused in frame with the 5′ end of gX, which encodes a signal sequence (17). An asterisk indicates the position of the polyadenylation site of gX. Plasmid pMZ72 contains the same deletions as pMZ69, pMZ68, and pMZ67 but lacks an HCV sequence. PgX, promoter of gX; Ul, unique long region; H, *Hin*dIII. (B) Generation of recombinant viruses M203, M204, M205, and control virus M206, by cotransfection of the E1-containing fragments or the control fragment lacking E1 with four overlapping subgenomic fragments. The origin of cosmid clones c-179, c-1, and c-443, which carry wild-type PRV fragments, has been described previously (23). Plasmid pMZ64, carrying a PRV fragment with an inactivated thymidine kinase gene, was obtained by cloning a 30-kbp *Bgl*II fragment isolated from the attenuated PRV strain 783 (5, 10), into the *Bgl*II site of plasmid vector pMZ20R. Parentheses indicate regions of the viral genome that were deleted.

genome of these viruses was verified by Southern blot analysis (data not shown). Northern blot analysis of RNA, isolated from SK-6 cells (7) infected with the recombinant viruses, revealed E1 transcripts of the expected size (data not shown). Furthermore, insertion of E1 in the viral genome did not influence growth of the viruses in cell culture (data not shown).

The expression of E1 by recombinant viruses was ana-

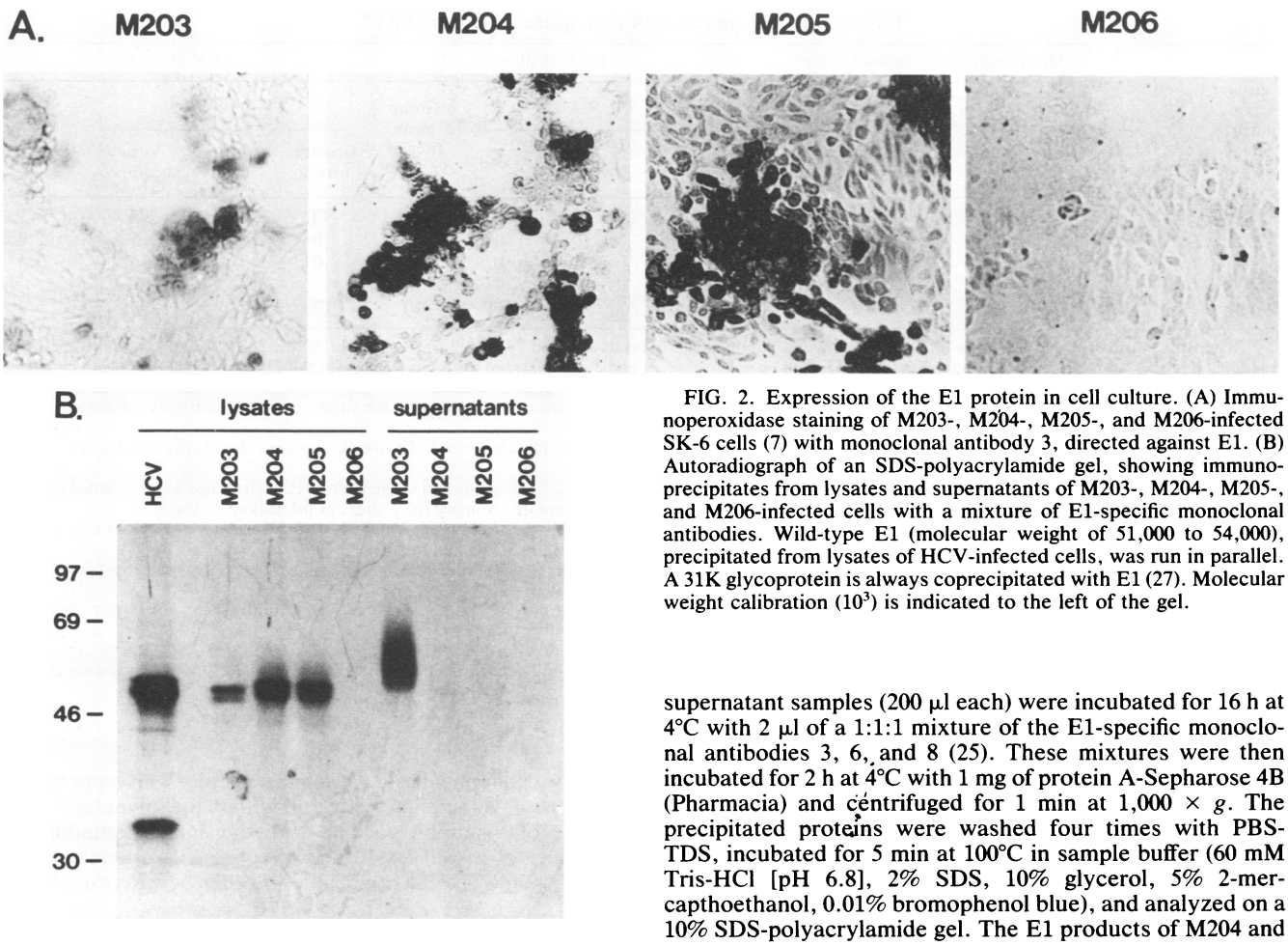

FIG. 2. Expression of the E1 protein in cell culture. (A) Immunoperoxidase staining of M203-, M204-, M205-, and M206-infected SK-6 cells (7) with monoclonal antibody 3, directed against E1. (B) Autoradiograph of an SDS-polyacrylamide gel, showing immunoprecipitates from lysates and supernatants of M203-, M204-, M205-, and M206-infected cells with a mixture of E1-specific monoclonal antibodies. Wild-type E1 (molecular weight of 51,000 to 54,000), precipitated from lysates of HCV-infected cells, was run in parallel. A 31K glycoprotein is always coprecipitated with E1 (27). Molecular weight calibration (10^3) is indicated to the left of the gel.

lyzed by immunoperoxidase staining of M203-, M204-, M205-, and M206-infected monolayers with a panel of monoclonal antibodies directed against four antigenic domains on E1 (Fig. 2A) (27, 28). Cells infected with the three PRV recombinants, but not with the control virus, stained with all monoclonal antibodies, indicating that the E1 variants harbored all the epitopes identified with these antibodies in the wild-type E1 protein. Cells infected with M204 and M205, expressing E1 with a transmembrane domain, stained much more intensely than cells infected with M203, expressing E1 without such a domain. The E1 products of M203, M204, and M205 were also analyzed by immunoprecipitation of lysates and supernatants of infected cells with a mixture of E1-specific monoclonal antibodies (Fig. 2B). Monolayers of 3×10^6 SK-6 cells were infected with HCV, M203, M204, M205, or M206 at a multiplicity of infection of 10. Cells were labeled with ^{35}S from 6 to 18 h after infection by incubation in 2 ml of cysteine-free Eagle basal medium supplemented with glutamine, antibiotics, 5% dialyzed fetal calf serum, and 100 μCi of [^{35}S]cysteine (Amersham) per ml. At 18 h after infection, cells and supernatants were harvested separately. Cells were lysed in 1 ml of PBS-TDS (1% [vol/vol] Triton X-100, 0.5% [wt/vol] Na-deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS] in phosphate-buffered saline). Lysates were sonicated and clarified by centrifugation for 10 min at 80,000 × *g*. Supernatants were clarified by centrifugation for 2 h at 80,000 × *g*. Lysate samples (100 μl each) or supernatant samples (200 μl each) were incubated for 16 h at 4°C with 2 μl of a 1:1:1 mixture of the E1-specific monoclonal antibodies 3, 6, and 8 (25). These mixtures were then incubated for 2 h at 4°C with 1 mg of protein A-Sepharose 4B (Pharmacia) and centrifuged for 1 min at 1,000 × *g*. The precipitated proteins were washed four times with PBS-TDS, incubated for 5 min at 100°C in sample buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercapthoethanol, 0.01% bromophenol blue), and analyzed on a 10% SDS-polyacrylamide gel. The E1 products of M204 and M205 were recovered only from cell lysates, whereas the E1 product of M203 was also detected in the supernatant. Evidently, the lack of a C-terminal hydrophobic region resulted in the release of this protein into the medium, which may explain the reduced immunostaining of the M203-infected cells. All recombinant E1 proteins precipitated from cell lysates appeared as a doublet, as did the wild-type E1 protein. The E1 product of M203 had an apparent molecular weight of 48,000 to 51,000, which is slightly smaller than that of the wild-type E1 (51,000 to 54,000). The E1 products of both M204 and M205 appeared similar in size to that of wild-type E1. The E1 protein encoded by M205 was expected to have a higher molecular weight than the E1 protein encoded by M204. A cellular protease probably trims the protein encoded by M205 and possibly also trims the protein encoded by M204, to similar-sized products. [^3H]glucosamine labeling studies showed that all E1 products were glycosylated (data not shown). The E1 product of M203 precipitated from the cell supernatant had a lower mobility and was more diffuse on SDS-polyacrylamide gel electrophoresis than the protein precipitated from the cell lysate, suggesting that it was glycosylated differently.

The immunogenicity and protective capacity of the recombinant viruses were examined in pigs (Table 1). Groups of four specific-pathogen-free (SPF) pigs were inoculated twice intramuscularly with 2×10^7 PFU of M203, M204, M205, or M206. No signs of disease or shedding of recombinant virus were observed after inoculation, and the animals invariably developed high titers of neutralizing antibodies against PRV.

TABLE 1. Immunization of pigs against HCV and PRV[a]

Virus strain inoculated	No. of pigs	Mean neutralizing antibody titer[b]							HCV challenge[c]				PRV challenge			
		HCV			PRV				No. of pigs			Group mean titer of viremia[d]	No. of days of:			Group mean titer of virus shedding[e]
		Day −2	Day 28	Day 42	Day −2	Day 28	Day 42	Day 63	With disease	With viremia	Dead		Growth arrest[f]	Fever[g]	Virus shedding[h]	
M203	4	0	0	1.9	0	1.5	4.3	5.2	3	3	0	2.8	NT	NT	NT	NT
M204	4	0	1.7	3.3	0	1.6	5.2	4.3	0	1	0	−0.5	0	0	5	2.0
M205	4	0	1.4	3.5	0	1.7	4.4	3.8	0	0	0	0	0	0	6	3.4
M206	4	0	0	0	0	2.2	4.2		4	4	4	5.4				
None	4	NT	NT	0	NT	NT	0	0	NT	NT	NT	NT	17	6	10	5.4

[a] Groups of four SPF pigs (12 weeks old) were inoculated intramuscularly on days 0 and 28 with 2×10^7 PFU of M203, M204, M205, or M206. To record shedding of virus, oropharyngeal fluid samples were collected on days −2, 3, 5, and 7 and analyzed for the presence of PRV (3). On day 42, pigs were challenged intranasally with 100 50% lethal doses of HCV strain Brescia 456610 (20, 26). On day 68, pigs inoculated with M204 and M205 and four SPF control pigs were challenged intranasally with 10^5 PFU of the virulent PRV strain NIA-3 (1, 2). Daily, animals were observed for signs of disease and body temperatures were measured. NT, not tested.

[b] The neutralizing antibody titer is expressed as the $\log_{10}$ of the reciprocal of the serum dilution neutralizing 100 50% tissue culture infective doses of HCV or PRV in 50% of the cultures (2, 19).

[c] Fever, anorexia, leukopenia, and nervous symptoms were regarded as signs of disease. Leukopenia and viremia after HCV challenge were recorded by taking heparinized blood samples on days 42, 45, 47, 49, 52, and 56, counting the leukocytes, and determining HCV titers in the leukocyte fractions.

[d] The mean titer of viremia per group is expressed as the $\log_{10}$ of the mean virus titer (50% tissue culture infective dose) in all leukocyte fractions taken on days 47, 49, 52, and 56.

[e] Mean titer of virus shedding per group is expressed as the $\log_{10}$ of the mean virus titer (PFU per milliliter) in all oropharyngeal fluid samples taken on days 69 to 78.

[f] Growth arrest is expressed as the number of days the group needed to regain their mean weight at postchallenge day 1 (in this experiment, day 69) (2). Pigs were weighed on days 66, 69, 71, 73, 76, 78, 80, 83, 85, and 87. None of the pigs died.

[g] Fever is expressed as the number of days during which the mean body temperature of animals of the group was above 40°C (2).

[h] Virus shedding is expressed as the number of days during which one or more animals of the group shed virus (2). Oropharyngeal fluid samples were collected on day 67 and daily from days 69 to 78 and analyzed for the presence of PRV (3).

All animals in the control group, inoculated with E1-negative M206, died when challenged intranasally with a lethal dose of HCV. The four animals inoculated with M203 developed low titers of neutralizing antibodies against HCV and were partly protected upon challenge; three animals became viremic and developed severe disease, but all survived. All eight pigs inoculated with M204 or M205 developed high titers of neutralizing antibodies against HCV and were completely protected against hog cholera. One of these pigs developed a low level of viremia. To detect transmission of HCV, a susceptible pig was housed from days 44 to 63 with each of the groups inoculated with M204 and M205 and monitored for signs of HCV infection and viremia in the same way as the challenged pigs. No transmission of HCV to the susceptible pigs was observed, and blood samples taken at day 84 were seronegative for HCV. The two groups inoculated with M204 and M205 and a control group of four SPF pigs were also challenged with virulent PRV. The animals of the control group developed severe pseudorabies, marked by weight loss and fever, and shed large amounts of virus. In contrast, the vaccinated animals showed no signs of disease and shed less virus.

We conclude from these data that vaccination of pigs with PRV recombinants expressing HCV glycoprotein E1 with a transmembrane domain protects pigs against both pseudorabies and hog cholera. The results clearly define E1 as an antigen that is sufficient to elicit a protective immune response against hog cholera. The presence of the hydrophobic region at the C terminus of E1 is required to obtain complete protection. However, the mechanism underlying this effect of the transmembrane domain is not known and is one of the aspects that need further investigation.

The finding that an attenuated PRV strain can be used as a vector to protect against another pathogen, while retaining its protective capacity against pseudorabies, makes attenuated PRV, which is used worldwide as a live vaccine, a highly economical and promising vector. This approach to vaccine development, which combines the advantage of the safety of a subunit vaccine with the antigen amplification capacity of a well-tested and effective live attenuated vaccine, should also be applicable to other herpesviruses.

We thank Catharinus Terpstra for continuing interest and helpful discussions, Nieke Oei for technical assistance, and Bart Hooft van Iddekinge for supplying labeled HCV proteins.

This work was supported by The Netherlands Technology Foundation, Duphar (a member of the Solvay Animal Health Group), and Intervet International BV (a subsidiary of AKZO).

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