Structural Proteins of Hog Cholera Virus Expressed by Vaccinia Virus: Further Characterization and Induction of Protective Immunity

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A cDNA fragment covering the genomic region that encodes the structural proteins of hog cholera virus (HCV) was inserted into the *tk* gene of vaccinia virus. Expression studies with vaccinia virus/HCV recombinants led to identification of HCV-specific proteins. The putative HCV core protein p23 was demonstrated for the first time by using an antiserum against a bacterial fusion protein. The glycoproteins expressed by vaccinia virus/HCV recombinant migrated on sodium dodecyl sulfate-gels identically to glycoproteins precipitated from HCV-infected cells. A disulfide-linked heterodimer between gp55 and gp33 previously detected in HCV-infected cells was also demonstrated after infection with the recombinant virus. The vaccinia virus system allowed us to identify, in addition to the heterodimer, a disulfide-linked homodimer of HCV gp55. The vaccinia virus/HCV recombinant that expressed all four structural proteins induced virus-neutralizing antibodies in mice and swine. After immunization of pigs with this recombinant virus, full protection against a lethal challenge with HCV was achieved. A construct that lacked most of the HCV gp55 gene failed to induce neutralizing antibodies but induced protective immunity.

Hog cholera is a contagious disease of swine which occurs worldwide and leads to severe economic losses. The causative agent, hog cholera virus (HCV), is a member of the genus *Pestivirus* within the family *Togaviridae* (41). The genus comprises also bovine viral diarrhoea virus (BVDV) and border disease virus of sheep; the different pestiviruses are serologically related (10, 12, 30).

Molecular characterization of pestiviruses started recently and resulted in cloning and sequencing of BVDV and HCV genomes (7, 28, 29, 31). The pestivirus genome represents a single-stranded RNA of about 12.5 kb which is of positive polarity and comprises a single large open reading frame (ORF). In HCV, the ORF is translated into a hypothetical polyprotein of 3,898 amino acids which gives rise to the mature proteins by proteolysis (28, 29). With respect to strategy of translation and genome organization, pestiviruses resemble flaviviruses (5). As with flaviviruses, pestivirus structural proteins are encoded at the 5' genomic end (6, 36). In contrast to flaviviruses, pestiviruses probably possess four structural proteins, three of which are glycosylated (for HCV, gp44/48, gp33, and gp55). HCV gp55 or the analogous BVDV gp53 appears to be of major importance for neutralization of pestiviruses, since neutralizing antibodies are directed against this glycoprotein (3, 13, 25, 39, 40). It was recently demonstrated that HCV gp55 or BVDV gp53 forms disulfide-linked heterodimers with a putative transmembrane protein, HCV gp33 or BVDV gp25 (39).

For vaccination against hog cholera, live attenuated HCV strains are used. Their use, however, is restricted in many countries, among other reasons because infections with virulent virus cannot be clearly differentiated from immunization with vaccine virus. Obviously, a recombinant vaccine would be advantageous in this regard. We report here expression and immunization studies using recombinant

vaccinia virus/HCV constructs that contain different cDNA fragments covering the coding regions for the structural proteins of HCV.

MATERIALS AND METHODS

Cells and viruses. CVI, 143tk⁻, and PK15 cells were obtained from the American Type Culture Collection (Rockville, Md.). W. Schäfer (Max-Planck-Institut für Virusforschung, Tübingen, Federal Republic of Germany) kindly provided the pig lymphoma cell line 38A₁D (37). Cell lines were grown in Dulbecco modified Eagle medium with 10% fetal calf serum. Vaccinia virus WR and the vaccinia virus *ts7* mutant were obtained from G. L. Smith (Cambridge, United Kingdom). HCV Alfort (1) was from B. Liess (Veterinary School, Hannover, Federal Republic of Germany).

Construction of recombination plasmids. Plasmids pGS62 core and pGS62-3.8 (Fig. 1) were constructed by standard procedures (26). The vaccinia virus recombination vector pGS62 (9) was obtained from G. L. Smith. Exonuclease III and nuclease S1 (both from Boehringer GmbH, Mannheim, Federal Republic of Germany) were used to shorten plasmid pH3.8 for generation of the deletion mutant (16). The oligonucleotide adaptors VacI and VacII were synthesized on a Biosearch 8700 DNA synthesizer (New Brunswick Scientific, Federal Republic of Germany) by the phosphoramidite method (2). Oligonucleotides were purified by using denaturing 20% polyacrylamide gels containing 7 M urea.

Recombinant vaccinia virus. CVI cells were infected with the thermosensitive vaccinia virus mutant *ts7* (14) at 33°C for 1 h at a multiplicity of infection of 0.5. After 2 h at 33°C, cotransfection of plasmid and vaccinia virus WR DNA was performed with a mammalian transfection kit as recommended by the supplier (Stratagene, Heidelberg, Federal Republic of Germany). After 48 h at 39.5°C, cells were harvested and the virus progeny was released by repeated freezing-thawing. For selection of thymidine kinase-negative

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phenotype, human 143tk⁻ cells were infected with this material and overlaid with Dulbecco modified Eagle medium containing 1% agarose and bromodeoxyuridine (100 µg/ml). After 2 days, distinct virus plaques were visible and plaque purification was performed. The vaccinia virus/HCV recombinants were termed VACcore, VAC3.8, and VAC3.8*.

Southern blot analysis. Vaccinia DNA was obtained from infected CVI cells by the quick-lysis method (32). The DNA was digested with *Hind*III, separated on a 0.8% agarose gel, and transferred to a Duralon membrane (Stratagene). Hybridization with pGS62core, labeled with [α -³²P]dCTP (3,000 Ci/mmol; Amersham Buchler, Braunschweig, Federal Republic of Germany) by nick translation (nick translation kit; Amersham Buchler), was carried out for 14 h at 68°C in hybridization solution (500 mM sodium phosphate [pH 6.8], 7% sodium dodecyl sulfate [SDS], 1 mM EDTA). Filters were washed at hybridization temperature twice for 15 min each time with 40 mM sodium phosphate (pH 6.8)–5% SDS–1 mM EDTA and twice for 15 min each time with 40 mM sodium phosphate (pH 6.8)–1% SDS–1 mM EDTA and exposed at –70°C to Kodak X-Omat AR films, using Agfa Curix MR 800 intensifying screens.

Neutralization assay. A given dilution of HCV was incubated with serial dilutions of antisera. PK15 cells were added and seeded in 96-well microtiter plates. After 3 days, the confluent monolayers were washed with phosphate-buffered saline, air dried, and fixed with 20% acetone in phosphate-buffered saline for 30 min. After incubation with a mouse monoclonal antibody (MAb) against HCV gp55 (39), a rabbit anti-mouse immunoglobulin fluorescein isothiocyanate conjugate (Dianova, Hamburg, Federal Republic of Germany) served for detection of HCV antigens in the cytoplasm.

Neutralization of vaccinia virus was determined in a plaque reduction assay in 24-well plates on CVI cells, using methylcellulose to inhibit virus spread.

Metabolic labeling of cells. A total of 10⁷ cells per ml were labeled for different time periods with 0.5 mCi of [³⁵S] methionine/cysteine or [³H]glucosamine (Amersham Buchler) per ml. The labeling medium contained either no cysteine and 1/20 of normal methionine content (³⁵S-amino acids) or 20 mM fructose instead of glucose ([³H]glucosamine). After the labeling period, the cells were stored at –70°C. Labeling started always 24 h after infection.

Radioimmunoprecipitation and SDS-PAGE. Cell extracts were prepared as described earlier (11) and incubated with appropriate dilutions of antiserum or MAb. Precipitates were formed with cross-linked *Staphylococcus aureus* (19), analyzed by suitable SDS-polyacrylamide gel electrophoresis (PAGE) (21, 34), and processed for fluorography by using En³Hance (New England Nuclear, Boston, Mass.). The dried gels were exposed to Kodak XAR5 X-ray films at –70°C. The ¹⁴C-labeled high-molecular-weight standards were from Amersham Buchler.

Two-dimensional SDS-PAGE. Two-dimensional SDS-PAGE was performed essentially as described previously (15). Briefly, precipitates obtained from radioimmunoprecipitation were subjected to 7.5% SDS-PAGE under nonreducing conditions in tube gels. The second dimension was performed under either nonreducing or reducing conditions. For the nonreducing analysis, the tube gel was directly put on an 8.75% slab gel. For separation under reducing conditions, the tube gel was treated with equilibration buffer (62.5 mM Tris hydrochloride [pH 6.8], 8.7% glycerine, 5% β -mercaptoethanol, 2.3% SDS, 8 M urea) for 10 min at 95°C and then immobilized with 1% agarose on an 8.75% SDS-con-

taining slab gel. The gels were processed after electrophoresis for fluorography as described above.

Preparation of anti-G1 serum. Subcloning of an HCV-derived cDNA fragment coding for amino acids 1 to 55 of the HCV ORF into the expression vector pEX34 was done according to standard procedures (26). pEX34 is identical to the expression vector pEX31 (38) except for a deleted *Psr*I site in the ampicillin resistance gene; expression and enrichment of the bacterial fusion protein were done basically as described previously (38). Fusion protein was further purified by preparative SDS-PAGE and after electroelution injected subcutaneously into rabbits after emulsification in Freund adjuvant (complete for basis immunization and incomplete for booster injections).

Animal experiments. Mice were injected intraperitoneally with vaccinia virus WR or different vaccinia virus/HCV recombinants. For infection of pigs with vaccinia virus/HCV recombinants or parental vaccinia virus WR, different routes of injection (intradermal, intraperitoneal, and intravenous) were used. Sera from mice and pigs were tested 4 weeks after infection for neutralizing antibodies. The pigs were challenged 4 weeks after immunization with 5 $\times$ 10⁷ 50% tissue culture infective doses (TCID₅₀) (lethal dose) of HCV Alfort by nasal inoculation.

RESULTS

Core construct. According to the genome organization suggested for BVDV (6) and HCV (38), the amino acid sequence preceding the glycoproteins should represent the nucleocapsid protein or part thereof. For BVDV, a 20-kDa protein that originates from the 5' end of the ORF was identified (6). The respective HCV protein has not yet been demonstrated. Sequence analysis revealed that a stretch of 18 hydrophobic amino acids is present between the putative core coding sequence and the gene encoding HCV gp44/48. We reasoned that this stretch between amino acids 250 and 267 represents the signal sequence responsible for translocation of the glycoproteins. To express the putative core protein separately from other HCV proteins, the respective coding sequence without the following signal sequence was inserted into the vaccinia *tk* gene under control of the P7.5 promoter (24).

For construction of the plasmid for recombination, a 0.8-kb *Hin*FI–*Bgl*II fragment (nucleotides 373 to 1117 of the HCV sequence [28]) isolated from HCV cDNA clone pHCK11 was subsequently inserted together with *Bam*HI–*Hin*FI adaptor *Vac*I and *Bgl*II–*Eco*RI adaptor *Vac*II (Fig. 1) into a pEMBL18⁺ plasmid, resulting in plasmid pHCCore. Ligation of the HCV cDNA fragment with adaptor *Vac*II to the *Eco*RI site of the vector DNA resulted in generation of an in-frame translational stop codon. The insert of pHCCore encompassed the sequences encoding amino acids 1 to 251 of the HCV polyprotein.

According to sequence analysis, HCV does not provide a sequence for efficient translation initiation. Therefore, the sequence CCACC, which is compatible with Kozak's suggestion (20, 43), was provided within the sequence of adaptor *Vac*I between the *Bam*HI-compatible cloning site and the authentic ATG (nucleotides 364 to 366 of the HCV sequence [28]) of the single ORF. The entire HCV insert was isolated after *Bam*HI–*Eco*RI digest and ligated into the vaccinia virus recombination vector pGS62 (9), giving rise to pGS62core (Fig. 1).

Construction of pGS62-3.8. For expression of the HCV-encoded structural proteins by vaccinia virus, a construct

FIG. 1. Construction of plasmids for generation of HCV recombinants. The plasmid containing the core coding sequence was generated by insertion of the *HinfI*-*BglI* fragment of pHC K11 (same as HCV cDNA clone 4.0 described previously [28]) together with the indicated oligonucleotide adaptors VacI and VacII into a pGem3 plasmid. For technical reasons, the 5' and 3' parts of the construct were built up independently, giving rise to plasmids pHCco and pHC re (5' and 3' fragments), respectively. After digestion with *HindIII* and *StuI*, the resulting plasmid pHC core served as a vector for the *HindIII*-*EcoRI* fragment of pHC K11. The *EcoRI* site was blunt ended by treatment with mung bean nuclease to obtain a stop codon after ligation into the *StuI* site of pHC core. Finally, the inserts of pHC core and pHC 3.8 were ligated after *BamHI*-*EcoRI* digestion into vaccinia virus recombination vector pGS62, which was cleaved accordingly.

encompassing almost 4 kb of the HCV ORF was generated. This sequence covered the genes for the three glycoproteins as well as the putative core protein (6, 36). A 3.4-kb *HindIII*-*EcoRI* fragment was isolated from pHC K11 and ligated into the pHC core plasmid digested with *HindIII*-*StuI* (Fig. 1). To obtain a translational stop codon at the 3' end of the HCV cDNA fragment, the *EcoRI* site was blunt ended by using mung bean nuclease prior to cloning. The resulting insert (up to nucleotide 4000 of the HCV sequence [28]) was isolated after *BamHI*-*EcoRI* cleavage and ligated into *BamHI*-*EcoRI*-digested pGS62, giving rise to pGS62-3.8 (Fig. 1).

Generation of recombinant vaccinia viruses. For generation of vaccinia virus/HCV recombinants, the respective plasmids were cotransfected with genomic vaccinia virus WR DNA into CVI cells that had been previously infected with the thermosensitive vaccinia mutant *ts7* at the permissive temperature (33°C) (14). After selection of the virus progeny for thymidine kinase-negative phenotype on human 143tk⁻ cells, recombinant vaccinia virus was identified by hybridization using HCV-specific probes; recombinant viruses were termed VACcore and VAC3.8, respectively. To check

whether recombination had taken place within the vaccinia virus *tk* gene, viral DNA was isolated, digested with *HindIII*, and separated on agarose gels. After Southern transfer and hybridization with *tk* sequences, two fragments of 4.4 and 1.2 kb (VACcore) or 7.3 and 1.2 kb (VAC3.8) instead of the 4.9-kb *HindIII* J fragment (WR) were detectable, indicating correct insertion of the HCV sequences (Fig. 2).

Identification of the HCV core protein. To identify the putative core protein, radioimmunoprecipitation analyses were performed. Pilot experiments indicated that incubation of the available polyspecific sera against HCV (autologous goat serum [33] or pig sera [unpublished data]) with extracts from VACcore-infected cells resulted in barely detectable specific signals. Therefore, an antiserum directed against a bacterial fusion protein encompassing amino acids 1 to 55 of the HCV ORF was prepared in a rabbit (anti-G1 serum). CVI cells were infected with VACcore and VAC3.8 and then labeled metabolically with a mixture of [³⁵S]cysteine/methionine, and cell extracts were incubated with preimmune rabbit serum and rabbit antiserum. To allow direct comparison, an extract prepared from HCV-infected 38A₁D cells labeled accordingly was included.

FIG. 2. Southern blot analysis of vaccinia virus-HCV recombinants VACcore and VAC3.8. Vaccinia virus DNA was obtained from infected CVI cells by the quick-lysis method (32) and separated on a 0.8% agarose gel after *Hind*III digest. A radiolabeled pGS62 core plasmid served as a probe. Lanes 1, WR strain DNA; 2, VACcore DNA; 3, VAC3.8 DNA. The size of the *Hind*III J fragment of the parental WR strain DNA is indicated (4.9 kb). Together with the 1.2-kb fragment (bottom arrowhead) resulting from an internal *Hind*III site of the HCV sequence, the recombinant *Hind*III J fragments change in size: VACcore (4.4 kb) (middle arrowhead) and VAC3.8 (7.2 kb) (top arrowhead).

The antiserum specifically recognized a protein with an apparent molecular size of 23 kDa (HCV p23) in extracts of cells infected with VACcore (Fig. 3, lane 4), VAC3.8 (lane 5), and HCV (lane 6). Proteolytic processing between the putative HCV core protein and gp44/48 was apparently identical in HCV- and VAC3.8-infected cells. The VACcore construct probably comprises the complete p23, since its migration by SDS-PAGE was identical to that of p23 from HCV-infected cells. In comparison to infection with HCV, however, expression of the protein was apparently stronger in cells infected with the vaccinia virus/HCV recombinants. Metabolic labeling, immunoprecipitation, and SDS-PAGE with noninfected 38A₁D cells as well as WR-infected CVI cells did not lead to any specific signals (data not shown). HCV p23 most likely represents the antigen analogous to BVDV p20 (6).

HCV-specific glycoproteins expressed by VAC3.8. HCV-specific proteins expressed by VAC3.8 were also characterized by radioimmunoprecipitation and SDS-PAGE. Infection of CVI cells with VAC3.8 or vaccinia virus WR was followed by metabolic labeling with a mixture of [³⁵S] cysteine/methionine. To include authentic glycoproteins, 38A₁D cells were infected with HCV and labeled accordingly. A polyspecific goat anti-HCV serum (33) and an MAb against HCV gp55 (MAb A18) (39) served as immunological reagents. Precipitates obtained after incubation of the goat

FIG. 3. Demonstration of the putative HCV core protein. CVI cells were infected with VACcore (lanes 1 and 4) and VAC3.8 (lanes 2 and 5) and labeled metabolically with [³⁵S]methionine/cysteine. In parallel, 38A₁D cells (lanes 3 and 6) were infected with HCV and labeled accordingly. For immunoprecipitation, preimmune rabbit serum (lanes 1 to 3) and rabbit antiserum (anti-G1) (lanes 4 to 6) were used; the latter was raised against a bacterial fusion protein encompassing HCV amino acids 1 to 55. The precipitates were separated by 12% SDS-PAGE (34). K, Kilodaltons.

anti-HCV serum with extracts from either VAC3.8- or HCV-infected cells led to signals corresponding to molecules with apparent molecular sizes of about 46 and 55 kDa (Fig. 4, lanes 3 and 4). Incubation of the extracts from VAC3.8-infected cells with MAb A18 resulted in a dominant 55-kDa protein band (lane 6). CVI cells were also metabolically labeled with [³H]glucosamine after infection with VAC3.8 (lanes 8 to 11). Sugar labeling should be advantageous for detection of HCV gp33 and also of HCV gp44/48, which are usually difficult to demonstrate after labeling with ³⁵S-amino acids, especially when polyspecific sera are used (33). While the goat anti-HCV serum precipitated molecules corresponding to the HCV-encoded glycoproteins gp33, gp44/48, and gp55 (Fig. 4, lane 8), MAb A18 led to detection of gp33 in addition to gp55 (lane 10). Actually, the MAb allowed detection of HCV gp33 upon labeling with ³⁵S-amino acids (faint band in lane 6). As recently demonstrated, coprecipitation of HCV gp33 is due to formation of a disulfide-linked heterodimer that decays after reduction to gp33 and gp55 (39). An additional band with an apparent molecular size of 90 to 100 kDa detected in HCV-infected cells after nonreducing SDS-PAGE (39) has not been further analyzed.

Analysis of disulfide-linked gp55 complexes. On the basis of these data, we reasoned that vaccinia virus-vectored HCV structural proteins were basically authentic with respect to proteolytic processing, molecular weight, glycosylation, and formation of heterodimers. In comparison with an infection of tissue culture cells with HCV, however, the proteins are apparently provided in larger amounts by recombinant vaccinia virus. Two-dimensional SDS-PAGE was used for further analysis of disulfide-linked complexes. VAC3.8-infected cells were metabolically labeled with [³⁵S]methionine/cysteine and incubated with MAb A18 (39). SDS-PAGE of the precipitates was performed under nonreducing conditions for the first dimension and under either nonreducing or

FIG. 4. Immunoprecipitation assay for analysis of HCV-specific proteins expressed by VAC3.8. CVI cells were infected with VAC3.8 (lanes 4 to 11) or vaccinia virus WR (lanes 1 and 2) and labeled metabolically with a mixture of [35 S]cysteine/methionine (lanes 1 to 7) and [3 H]glucosamine (lanes 8 to 11). Cell extracts were incubated with polyspecific goat anti-HCV serum (lanes 1, 4, and 8), goat preimmune serum (lanes 2, 5, and 9), MAb A18 (lanes 6 and 10), and MAb anti-FMDV (control) (lanes 7 and 11). In lane 3, a precipitate from extracts of HCV-infected 38A₁D cells with goat anti-HCV serum was loaded onto the gel. Marked by arrowhead are the HCV-specific glycoproteins gp55, gp44/48, and gp33. Immunoprecipitates were analyzed by 10% SDS-PAGE (21) and visualized by fluorography. The figure represents a composite from one gel. K, Kilodaltons.

reducing conditions for the second dimension. Nonreducing conditions in both dimensions led to identification of molecules with apparent molecular sizes of 55 (gp55), 75 (gp75), and 90 to 100 (gp95) kDa (Fig. 5a), similar to earlier results obtained after infection with HCV (39). Reduction of gp75 and SDS-PAGE in the second dimension resulted in molecules of 55 and 33 kDa (Fig. 5b). In agreement with previous reports, this result reflects the heterodimeric nature of gp75, which is composed of disulfide-linked gp55 and gp33. Interestingly, gp95 gave rise to a single 55-kDa band after reduction. Thus, in both the vaccinia virus recombinant- and HCV-infected cells (data not shown), gp55 formed intracellularly disulfide-linked homodimers as well as heterodimers. We have no indication for heterotrimers between gp55 and gp33 as described by Wensvoort et al. (40).

Immunization studies with VAC3.8. Vaccinia virus recombinants are well suited for immunization experiments, since the vector possesses broad host range and low virulence. To check whether the recombinants induced an immune response against HCV in vivo, mice were immunized with VAC3.8, VACcore, and vaccinia virus WR. Groups of mice

(three mice per group) were injected intraperitoneally with 2×10^7 PFU of VAC3.8 and VACcore; a control group was injected with 5×10^6 PFU of WR. Four weeks after infection, titers of neutralizing antibodies were determined. Sera from mice immunized with VAC3.8 contained HCV-neutralizing antibodies (Table 1). As one might expect, immunization with either VACcore or WR did not lead to anti-HCV antibodies detectable by neutralization assays. Mice from all three groups developed vaccinia virus-neutralizing antibodies (1:32).

Because virus neutralization and protection against disease may not always correlate (17, 35, 42), we wished to conduct immunization studies with pigs, which represent the only natural host of HCV. The approach allowed monitoring of protection against a challenge with a lethal dose of HCV. In the first experiment, two pigs were infected intradermally, intraperitoneally, and intravenously with VAC3.8 (5×10^7 PFU by each route). As a control, one pig was immunized with the parental vaccinia virus WR strain. As in the experiment in mice, VAC3.8 was capable of inducing HCV-neutralizing antibodies (Table 2). Four weeks after the single immunization, the pigs were challenged with a lethal dose of 5×10^7 TCID₅₀ of HCV Alfort intranasally, which corresponds to the natural route of infection. The WR-immunized pig developed typical symptoms of HC, which were already observed on day 5 after challenge infection, and was killed on day 12 in a moribund state. Both pigs immunized with VAC3.8 remained unaffected by the challenge infection for the following 21 days.

Construction of gp55 deletion mutant. The glycoprotein HCV gp55 or the analogous BVDV gp53 has become a major focus in pestivirus research because it induces virus-neutralizing antibodies (3, 13, 25, 39, 40). It was of interest to determine whether other structural proteins mediate neutralization and also whether the development of protective immunity depends on the presence of HCV gp55. To approach these questions, a mutant of VAC3.8 lacking most of the gp55-coding sequence was generated. About 1.4 kb at the 3' end of VAC3.8 was deleted by exonuclease III treatment of a *KpnI-EcoRI*-digested pHC3.8 plasmid (Fig. 1). The truncated insert was ligated into a pGS62 plasmid and gave rise to vaccinia virus recombinant VAC3.8*. The HCV sequence present in VAC3.8* stops at nucleotide 2589 (amino acid 742). According to the proposed processing sites between HCV gp33 and gp55 (29, 36), almost the entire gp55-coding sequence was deleted except for a residue of about 40 amino acids (Fig. 6).

The effect of the HCV gp55 deletion on the proteins expressed by VAC3.8* was first analyzed after radioimmunoprecipitation. CVI cells were infected with VAC3.8, VAC3.8*, and VACcore and labeled metabolically with [35 S]cysteine/methionine. Cell extracts were incubated with goat preimmune serum, goat anti-HCV serum, MAb A18 (anti-gp55), a control MAb against foot-and-mouth disease virus (FMDV), anti-G1 serum, and preimmune rabbit serum. An HCV gp55-related protein could not be demonstrated in extracts of VAC3.8*-infected cells (Fig. 7, lanes 5 to 8). The synthesis of gp44/48 was apparently unaffected by the deletion because this glycoprotein migrated identically to the one precipitated from cells infected with VAC3.8 (lanes 2 and 5). HCV gp33 was not precipitated by the goat anti-HCV serum (lane 5). However, gp33 of the original size could be detected after infection with VAC3.8* when antibodies against bacterial fusion 1 (36) were used (data not shown). This finding indicates that the cleavage between gp33 and the amino acids encoded by the truncated gp55 gene occurred at the correct

FIG. 5. Two-dimensional SDS-PAGE of HCV-specific glycoproteins expressed by VAC3.8. CVI cells were infected with VAC3.8 and labeled metabolically with [35 S]cysteine/methionine. The respective cell extract was subjected to radioimmunoprecipitation using MAb A18 and 7.5% SDS-PAGE in a tube gel without reduction of the sample. For the second dimension in panel a, the gel was loaded onto a 8.75% SDS-polyacrylamide gel, again using nonreducing conditions. Marked by arrowheads are HCV gp55, gp75, and gp95. (b) Two-dimensional SDS-PAGE using the same precipitates as in panel a. After separation in the first dimension, the tube gel was soaked in 5% β -mercaptoethanol and subjected to SDS-PAGE for the second dimension. Marked by arrowheads are HCV gp55 and gp33. K, Kilodaltons.

site. In addition, HCV p23 was specifically precipitated from cells infected with VAC3.8* (lane 10).

Immunization experiments with VAC3.8*. Two pigs were infected with VAC3.8*, three were infected with VAC3.8, and one was infected with WR according to the protocol described above; 4 weeks after infection, the titers of neutralizing antibodies against HCV as well as vaccinia virus were determined. As in the experiments performed with mice (Table 1), HCV-neutralizing antibodies were not detectable in sera from both pigs immunized with VAC3.8*, while titers of vaccinia virus-neutralizing antibodies were similar to those assayed after infection with VAC3.8 and WR. Obviously, induction of HCV-neutralizing antibodies depends largely or even exclusively on the presence of HCV gp55 (Table 2).

Four weeks after immunization with vaccinia virus recombinants, challenge experiments were performed by nasal inoculation of 5×10^7 TCID₅₀ of HCV Alfort. Whereas the pig infected with WR developed hog cholera and was killed at day 14 after challenge, the three VAC3.8-immunized pigs

again remained unaffected by the HCV infection. The pigs immunized with VAC3.8* were feverish on day 5 after challenge infection (40.8 and 41.0°C, respectively) but recovered completely within the next 2 days. In both pigs, no manifestation of hog cholera occurred within the following 21 days.

DISCUSSION

Expression of the part of the HCV ORF that encodes the structural proteins via vaccinia virus led to identification of four HCV-specific proteins. Three of these represent glycoproteins that have been previously detected in virus-infected cells (gp44/48, gp33, and gp55) (33) and whose genome localization has been determined (36). The three vaccinia

TABLE 1. Titers of HCV-neutralizing antibodies in mice after infection with vaccinia virus-HCV recombinants^a

Group	Virus	Titer	
		Day 21	Day 28
1	WR	<1:2	<1:2
2	VACCore	<1:2	<1:2
3	VAC3.8	1:32	1:64
4	VAC3.8*	<1:2	<1:2

^a Groups of mice (three animals per group) were infected by intraperitoneal inoculation with 2×10^7 PFU of recombinant vaccinia virus. Strain WR was administered at only 5×10^6 PFU because of putative higher virulence. Titers of neutralizing antibodies against HCV were determined for complete neutralization of virus assayed by immunofluorescence. Titers for all groups were <1:2 on day 0.

TABLE 2. Titers of HCV-neutralizing antibodies after infection of pigs with vaccinia virus recombinants^a

Pig no.	Virus	Titer on day 28	Protection ^b
34	WR	<1:2	—
28	WR	<1:2	—
35	VAC3.8*	<1:2	+
36	VAC3.8*	<1:2	+
26	VAC3.8	1:32	++
27	VAC3.8	1:64	++
37	VAC3.8	1:64	++
38	VAC3.8	1:16	++
39	VAC3.8	1:16	++

^a The animals were injected intravenously, intraperitoneally, and intradermally with 5×10^7 PFU (VAC3.8 and VAC3.8*) and 10^7 PFU (WR) (each route of inoculation). Titers of neutralizing antibodies against HCV were assayed by immunofluorescence. Titers for all animals were <1:2 on day 0. Four weeks after immunization, challenge infection was performed by nasal inoculation of 5×10^7 TCID₅₀ of HCV Alfort.

^b ++, Full protection (no clinical signs of disease); +, partial protection (development of fever followed by complete recovery); —, no protection.

FIG. 6. Relative positions of proteins encoded by vaccinia virus/HCV recombinants VACcore, VAC3.8, and VAC3.8*. The 3' end of the HCV insert in pH3.8 was shortened by using exonuclease III after digestion with *Kpn*I and *Eco*RI. Numbers at the ends of the bars refer to the last HCV-specific amino acids encoded by the recombinants. Other numbers refer to putative processing sites of the polyproteins (36) and indicate the assumed last amino acids of the respective proteins.

virus-vectored HCV glycoproteins migrated by SDS-PAGE as did the proteins induced by HCV. This finding indicates that proteolytic processing and glycosylation are authentic in comparison with HCV-infected cells.

As the first protein of the BVDV ORF, a 20-kDa molecule has been identified as the putative nucleocapsid protein (8). An antiserum against a bacterial fusion protein encompassing the amino-terminal part of the HCV polyprotein allowed identification of a 23-kDa protein which most likely represents the analogous HCV protein, since the serum precipitated p23 not only from VAC3.8- and VACcore-infected cells but also from HCV-infected cells. The VACcore construct encodes the first 251 amino acids of the HCV ORF. In pulse-chase experiments with HCV- and VAC3.8-infected

cells, a larger precursor of p23 could not be demonstrated (data not shown). For processing between the putative core protein and a putative signal peptide, we therefore suggest the site EKA (amino acids 248 to 250). In analogy to other virus systems, this cleavage may be due to an autoproteolytic activity (27). The calculated molecular size of p23 is about 28.1 kDa. The composition of amino acids has been referred to as a possible reason for the anomalous migration of BVDV p20 (6), and this argument may also apply for HCV p23.

With regard to the development of recombinant vaccines, most attention has been paid to virus structural proteins that induce neutralizing antibodies. So far, there is no agreement about the molecular composition of pestivirions, including the envelope protein(s). It is, however, generally assumed that the antigen which mediates virus neutralization, HCV gp55 and the analogous BVDV gp53, forms part of the virion. Participation of other proteins, i.e., p23, gp44/48, and gp33, in assembly of pestiviruses has yet to be demonstrated. Interestingly, both HCV gp55 and BVDV gp53 form intracellularly disulfide linked heterodimers with a putative transmembrane protein, HCV gp33 or BVDV gp25 (39). In addition to the heterodimer, the vaccinia virus system allowed the demonstration of a disulfide-linked gp55 homodimer which is also present in HCV-infected cells (data not shown) and in virions (unpublished data). The biological significance of the two dimeric forms remains to be investigated.

Immunization experiments with two different vaccinia virus/HCV recombinants addressed the role of HCV structural proteins with regard to the induction of virus-neutralizing antibodies as well as protective immunity. In mice and pigs, neutralizing antibodies against HCV were induced after infection of animals with VAC3.8. In addition, immunization with the same recombinant protected pigs against a lethal challenge with HCV. By using vaccinia virus as a vector, similar results have been obtained in other virus systems, especially in connection with expression of envelope glycoproteins (4, 23). Interestingly, both pigs immunized with the recombinant lacking most of the HCV gp55-coding sequence

FIG. 7. Comparison of HCV-specific proteins expressed by VAC3.8 (lanes 1 to 4, 9, and 12), VAC3.8* (lanes 5 to 8, 10, and 13), and VACcore (lanes 11 and 14). CVI cells were labeled with a mixture of [35 S]methionine/cysteine after infection with the respective vaccinia recombinant. For immunoprecipitation, goat anti-HCV serum (lanes 2 and 5), goat preimmune serum (lanes 1 and 6), MAb A18 (lanes 3 and 7), control MAb anti-FMDV (lanes 4 and 8), rabbit antiserum (anti-G1) (lanes 9 to 11), and preimmune rabbit serum (lanes 12 to 14) were used. The precipitates were separated by 12% SDS-PAGE (34). HCV-specific proteins gp55, gp44/48, gp33, and p23 are indicated by arrowheads. K, Kilodaltons.

(VAC3.8*) were at least partially protected against a lethal challenge, although HCV-neutralizing antibodies were not detectable. Thus far, the major glycoprotein of pestiviruses (HCV gp55 or BVDV gp53) is the only antigen that can be directly linked to virus neutralization. The protective immunity obtained after vaccination with VAC3.8 could be explained by the mere induction of neutralizing antibodies, but the partial protection following injection of VAC3.8* argues against such an exclusive explanation. Almost nothing is known at this point about cellular immunity against hog cholera (22). It will be important to determine the role of the other expressed proteins (p23, gp44/48, and gp33) in induction of immunity.

Because of the enormous economic losses, eradication or at least control of hog cholera is an important task that would be facilitated by a suitable vaccine. Vaccines against hog cholera represent live virus strains that were attenuated by passages in rabbits. Their use is prohibited in many countries. Vaccination interferes with the "test and slaughter" policy commonly followed during HCV outbreaks. Recombinant vaccines such as vaccinia virus/HCV would solve this problem because the presence or absence of antibodies directed against, for example, HCV nonstructural proteins could be used for a simple diagnostic test.

We do not, however, suggest vaccinia virus as a suitable vector in the field. The sporadic pathogenicity of vaccinia virus recombinants for nonvaccinated humans is a sufficient reason to inhibit further release of infectious vaccinia virus into the environment (18). Nevertheless, the knowledge gained from experiments with vaccinia virus recombinants will be very helpful for the development of other vector systems for expression of HCV protein(s) such as porcine poxvirus or herpesviruses (e.g., pseudorabies virus). The latter system would provide a recombinant vaccine for simultaneous immunization against two economically important diseases of pigs, Aujeszky's disease and hog cholera.

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