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Processing in the Pestivirus E2-NS2 Region: Identification of Proteins p7 and E2p7

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The pestivirus genome encodes a single polyprotein which is subject to co- and posttranslational processing by cellular and viral proteases. The map positions of all virus-encoded proteins are known with the exception of a hypothetical peptide (p?) which interlinks the glycoprotein E2 and the nonstructural protein NS2-3 approximately between amino acid positions 1060 and 1130. Expression studies with recombinant vaccinia viruses bearing a set of C-terminally truncated E2-p?-NS2-encoding sequences derived from a bovine viral diarrhea virus (BVDV) strain led to the identification of a minor fraction of E2 which had an increased molecular mass due to a C-terminal extension. This larger form of E2 (E2p7) was specifically recognized by an antiserum raised against the amino acid sequence from 1065 to 1125. In addition, the antibodies revealed a BVDV-encoded 7-kDa protein (p7) in infected cells. By radiosequencing it was determined that Val-1067 was the N-terminal amino acid of in vitro-synthesized p7. Analyses of BVDV and classical swine fever virus virions suggest that neither p7 nor E2p7 is a major structural constituent.

Pestiviruses are classified as a separate genus within the family *Flaviviridae*, which also includes flaviviruses and the hepatitis C virus group (22). Currently three pestivirus species are recognized, namely, bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV) of sheep. The genomes of pestiviruses are positive-stranded RNAs, usually of about 12,300 nucleotides, which encode polyproteins of about 4,000 amino acids (3, 4, 9). Entire or partial genomic sequences of numerous BVDV, CSFV, and BDV isolates have been determined (1, 2, 4, 7, 9, 11, 12), and their comparison demonstrated a high degree of sequence conservation among pestiviruses.

The virions of pestiviruses consist, together with the RNA, of four structural proteins, the nucleocapsid C protein and the envelope glycoproteins E^{rns}, E1, and E2 (20). Currently 11 pestiviral proteins have been identified as products of polyprotein processing which occurs co- and posttranslationally because of viral and host cell proteases. In the hypothetical polyprotein, the proteins are arranged in the order N^{pro}/C/E^{rns}/E1/E2/NS2/NS3/NS4A/NS4B/NS5A/NS5B (3, 5, 15, 16, 19) (these are designations proposed by the *Flaviviridae* study group of the International Committee on the Taxonomy of Viruses). The existence of an additional protein (p?) located between E2 and NS2 was recently hypothesized (14). The speculation arose because of a gap in the organization of the polyprotein which was left between the C terminus of CSFV E2 (14) and the estimated N terminus of the NS2 of BVDV NADL (5). Recently, a hydrophobic p7 protein of hepatitis C virus was demonstrated to be either part of unprocessed E2p7NS2, E2p7, or an individual protein of 7 kDa (8, 10).

BVDV E2 has two different C termini. During glycoprotein maturation, a thus-far-uncharacterized cleavage occurs at the C terminus of the E2 glycoprotein. For the positions of the C terminus of E2 and the N terminus of nonstructural NS2-3, only rough estimates were available. We wished to map the C-terminal border of E2 and to identify the putative processing product(s) between E2 and NS2. A set of recombinant vaccinia viruses was generated which express N^{pro}, C, E^{rns}, E1, E2, and serially decreasing portions of p? and NS2-3 of BVDV CP7. The resulting vaccinia virus recombinants, among them Vac_{1-1140} and Vac_{1-1116}, were named according to the expressed portions of the BVDV CP7 polyprotein. Analysis of the apparent molecular masses of the proteins expressed by the vaccinia virus recombinants revealed that the predomi-

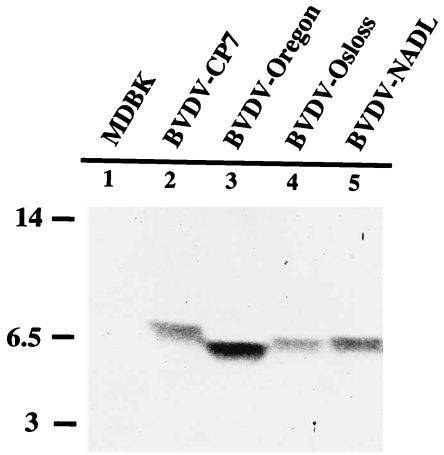

FIG. 1. Immunoblot detection of a 6- to 7-kDa polypeptide with antibodies generated against amino acids 1065 to 1125 of BVDV CP7; the respective BVDV cDNA fragment was expressed as a fusion protein with the N-terminal part of the MS2 polymerase, which is under the transcriptional control of the heat-inducible lambda pL promoter in plasmid pEX34 (17). pEX p7 was expressed in *E. coli* C600 carrying the repressor plasmid pc1857, and 200 μg of the purified protein was used for each immunization of laboratory rabbits. Lysates of cells infected with BVDV strains CP7 (6), Oregon (from Intervet, Netherlands), Osloss (12), and NADL (4) were subjected to SDS-PAGE (12% polyacrylamide) in a Tris/Tricine buffer system, transferred to nitrocellulose membranes, and probed with anti-p7 antibodies.

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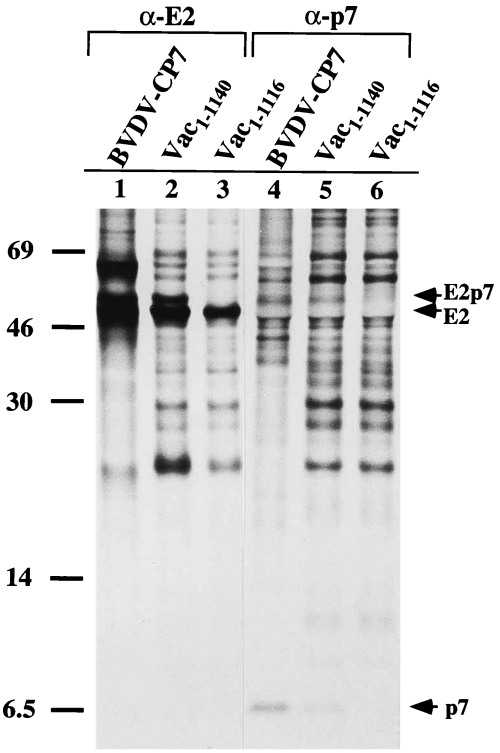

FIG. 2. Anti-p7 antibodies identify E2* as unprocessed E2-p7. A total of 1.5 × 10^6 MDBK cells were infected at a multiplicity of infection of 0.1 for 24 h. At 7 h postinfection, the growth medium was replaced with Dulbecco's modified Eagle's medium without methionine, and 500 μCi of [^{35}S]methionine was added 30 min later. CVI (1 × 10^6) cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 5 and labeled with 500 μCi of [^{35}S]methionine for 16 h beginning at 4 h postinfection. Extracts from cells infected with BVDV CP7, $Vac_{1–1140}$, or $Vac_{1–1116}$ were incubated with anti-BVDV E2 (α-E2) (a mixture of monoclonal antibodies provided by E. Weiland, Tübingen) and anti-p7 (α-p7) antibodies. Afterwards, the extracts were incubated with formaldehyde-fixed *Staphylococcus aureus* cells. Precipitates were subjected to SDS-PAGE. Gels were treated with En^{3}Hance (NEN) for fluorography. The positions of molecular mass markers (sizes are in kilodaltons) are shown on the left.

nant form of E2 (53 kDa) was not notably affected by the truncations (data not shown). A minor E2 species with an increased molecular mass (58 kDa), termed E2*, could only be observed in extracts from cells infected with BVDV or $Vac_{1–1140}$; it was absent from those infected with $Vac_{1–1116}$ (data not shown). To exclude the possibility that a variable degree of glycosylation accounted for the formation of E2*, immunoprecipitated material was treated with *N*-glycopeptidase F to fully remove the carbohydrate side chains. Precipitates from $Vac_{1–1116}$-infected cells yielded a single 41-kDa protein, while deglycosylation of precipitates from $Vac_{1–1140}$- as well as BVDV-infected cells resulted in the formation of two bands with apparent molecular masses of 41 and 46 kDa (data not shown). The results suggest that the protein backbone of E2* is extended compared with that of E2 and that the additional amino acids are located at the C terminus.

Demonstration of p7. The bulk of the E2 glycoprotein is in the 53-kDa form, while E2* accounts for only a minor fraction. The generation of E2 probably results from proteolytic processing which does not occur with E2*. To identify the putative cleavage product, antibodies were raised against amino acids 1065 to 1125 of the BVDV CP7 polyprotein (see Fig. 6B). A cDNA fragment encoding this peptide was fused to a fragment of the MS2 polymerase gene, and the fusion protein was expressed in *Escherichia coli*; the purified fusion protein served for immunization of two rabbits. The antibody response was poor, probably because of the hydrophobic nature of the peptide. Seven booster injections were needed to obtain an antiserum, which is referred to as anti-p7.

Extracts of cells infected with different BVDV strains (NADL, Oregon, Osloss, and CP7) were tested for their reactivity with the anti-p7 antibodies (Fig. 1). All BVDV isolates reacted with the antiserum, while the apparent molecular masses of the proteins differed slightly among the tested strains. Analysis of the set of vaccinia virus-BVDV recombinants with the anti-p7 antibodies resulted in detection of p7 in cells infected with $Vac_{1–1140}$ but not in cells infected with

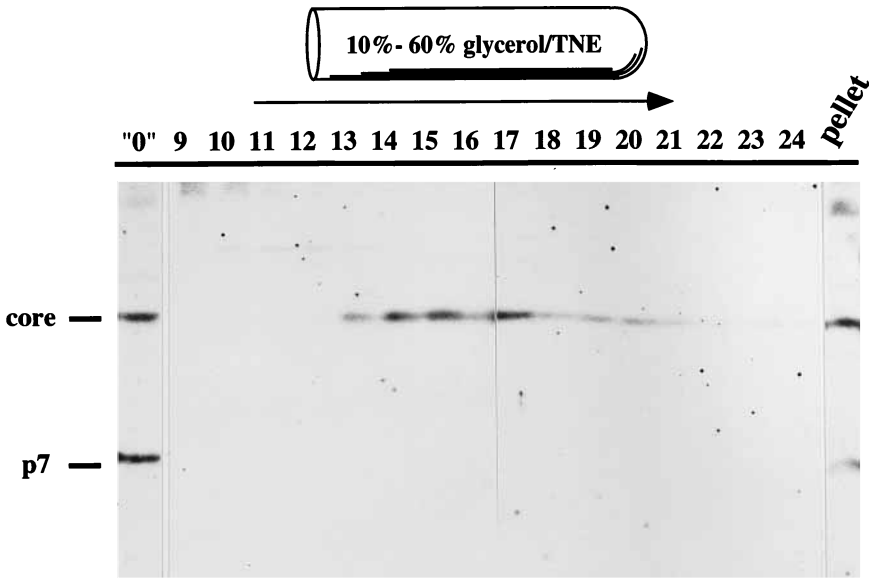

FIG. 3. p7 does not copurify with BVDV virions. Virus from the culture medium of BVDV Oregon-infected MDBK cells was precipitated with polyethylene glycol 8000 and further concentrated by ultracentrifugation. Pelleted virions ("0") were layered on top of a 10 to 60% glycerol–TNE gradient and centrifuged for 1 h at 30,000 rpm in an SW55 rotor. A total of 30 fractions were collected from the bottom and analyzed by immunoblotting with anti-p7 and anti-C antibodies (20). The nitrocellulose membrane was blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.05% Tween 20 for 1 h. The anti-p7 antibodies were used at a dilution of 1:500, and the anti-C antibodies were used at a dilution of 1:5,000. The choice of the blocking substance proved to be critical since p7 could not be detected after the filter was immersed in 2.5% skim milk–PBS. For revelation of a secondary signal, antibodies conjugated to alkaline phosphatase or peroxidase were used as recommended by the suppliers. A light-emitting detection system (ECL; Amersham) was used in combination with peroxidase-labeled antibodies according to the manufacturer's instructions.

Vac_{1-1116} (Fig. 2). The anti-p7 antibodies specifically reacted with E2* present in cells infected with BVDV, Vac_{1-1212}, or Vac_{1-1140}, which identifies E2* as the fusion protein E2p7 (Fig. 2).

p7 is not a major structural component of the virion. The anti-p7 antibodies enabled us to address whether p7 and/or E2p7 is an integral part of the virion. Pestiviruses remain cell associated, and virions in the culture medium are sparse. In addition, pestiviruses are difficult to purify by their physical properties since their density and sedimentation constants are similar to those of various vesicular components of the host cell (13). Sufficient amounts of cytopathogenic BVDV can be harvested after virus-induced disintegration of the monolayer. Unfortunately, enrichment of virions by differential centrifugation yielded virus preparations that were heavily contaminated with cellular debris and nonstructural proteins such as NS3. By using linear glycerol gradients, we found that zonal-rate centrifugation gave a sufficient degree of virus purification to at least address whether a protein copurifies with virus particles. For this purpose, virus from 1 liter of culture medium was concentrated and applied to a linear 10 to 60% glycerol–Tris-buffered sodium chloride-EDTA solution (TNE) gradient. Thirty fractions were collected and analyzed by immunoblotting (Fig. 3). Intact virus particles peaked in fractions 14 to 17, as determined by immunoelectron microscopy (data not shown). The blots were analyzed with anti-p7 and anti-CSFV capsid protein antibodies (20). The latter were used as an indicator of the position of virions along the gradient; in pilot experiments it was determined that in gradients, infectivity colocalizes accurately with the capsid protein (data not shown). p7 present in the starting material (Fig. 3) was detected after centrifugation at the bottom of the tube in the pellet, which mostly consisted of cellular debris. Even long exposures of the blots did not indicate the presence of p7 in fractions 13 to 20. In accordance with earlier analyses of the molecular compositions of pestiviruses (20), we reason that p7 is at least not a major constituent of the virion.

The question of whether E2p7 represents a structural protein could only be answered indirectly. Western blot (immunoblot) analyses with BVDV virions and anti-p7 antibodies gave no evidence for the presence of E2p7 in viral particles. This result, however, should be validated by the use of antibodies which detect E2 in a Western blot. Unfortunately, none of the anti-BVDV E2 antibodies available to us is reactive after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Also, the amounts of metabolically labeled antigens from virions were insufficient for immunoprecipitation analysis. We therefore turned to the CSFV system, the system in which the desired antibodies have been prepared. Analogous to the situation described for BVDV, two forms of E2 could be detected in CSFV-infected cells which vary in their protein backbones by about the size of the p7 peptide. Since CSFV grows to reasonable titers without an apparent cytopathic effect, we considered the pelleted virions clean enough to assess if they also contain different forms of E2. A comparison of the molecular mass of E2 from infected cells with that of E2 from virions required complete deglycosylation because the molecular masses contributed by carbohydrate chains in the two proteins are significantly different (20). E2 from infected cells clearly comprises two different protein backbones, while E2 from virions is exclusively in the smaller form (Fig. 4).

Generation of p7 requires microsomal membranes. To define the biosynthesis of p7 and E2p7 in more detail, processing was studied by in vitro translation. A cDNA served as the template for mRNA transcription in which the sequences encoding E2p7NS2 (BVDV amino acids 693 to 1588) were placed downstream from an SP6 promoter. For efficient translocation, a preprolactin cDNA encoding amino acids 1 to 53 was fused to the 5′ end of the E2 gene. RNA transcribed from the respective plasmid was used to direct in vitro translation in a rabbit reticulocyte lysate (Fig. 5). Addition of microsomal membranes allowed the generation of the individual products E2, E2p7, p7, and NS2, while in the absence of the membranes, only the unglycosylated precursor preprolactin-E2p7NS2 appeared. Thus, cleavage between E2 and p7 as well as between

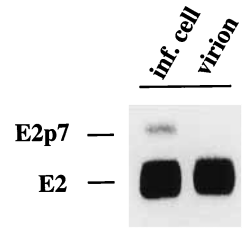

FIG. 4. E2p7 is not detectable in CSFV virions. Pelleted viruses and extract from CSFV-infected (inf.) pig lymphoma cells ($38A_1D$) were treated with *N*-glycopeptidase F and analyzed by immunoblotting with monoclonal antibody A18 (anti-E2) (21). E2p7 is present in CSFV-infected cells but not in pelleted virions.

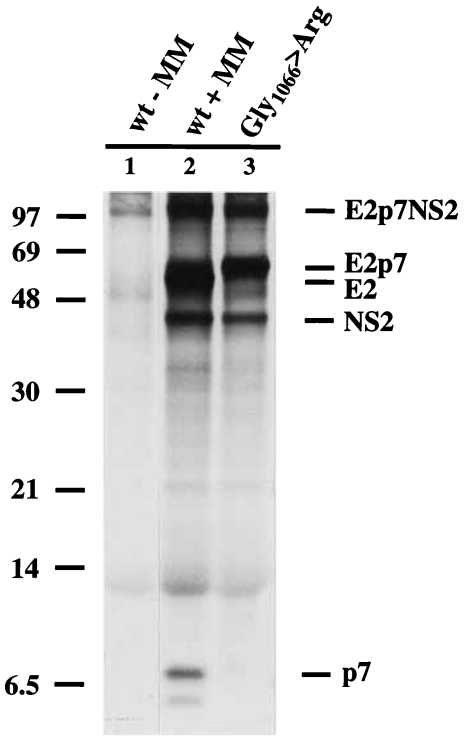

FIG. 5. Generation of p7 depends on the presence of microsomal membranes. The plasmid used for the generation of SP6 transcripts was obtained by cloning a cDNA fragment encoding amino acids 693 to 1588 of BVDV CP7 (restricted with *Kpn*I and *Bam*HI and treated with Klenow fragment) into the *Sma*I site of pRN654 (derived from pRN653 [18]), downstream of and in frame with the coding sequence for amino acids 1 to 53 of preprolactin. Translation of the synthetic mRNA encoding $preprolactin_{1-53}$-E2p7NS2 was carried out in reticulocyte lysate in the presence of [^{35}S]methionine with (+MM) or without (−MM) canine microsomal membranes. While only minor amounts of E2p7 can be detected with wild-type (wt) E2p7NS2, mutagenesis of Gly-1066 to Arg abolished the cleavage which generates E2 and p7 (lane 3). The uncleaved precursor, E2p7NS2, and the resulting processing products are indicated at the right margin. The positions of molecular mass markers (sizes are in kilodaltons) are on the left.

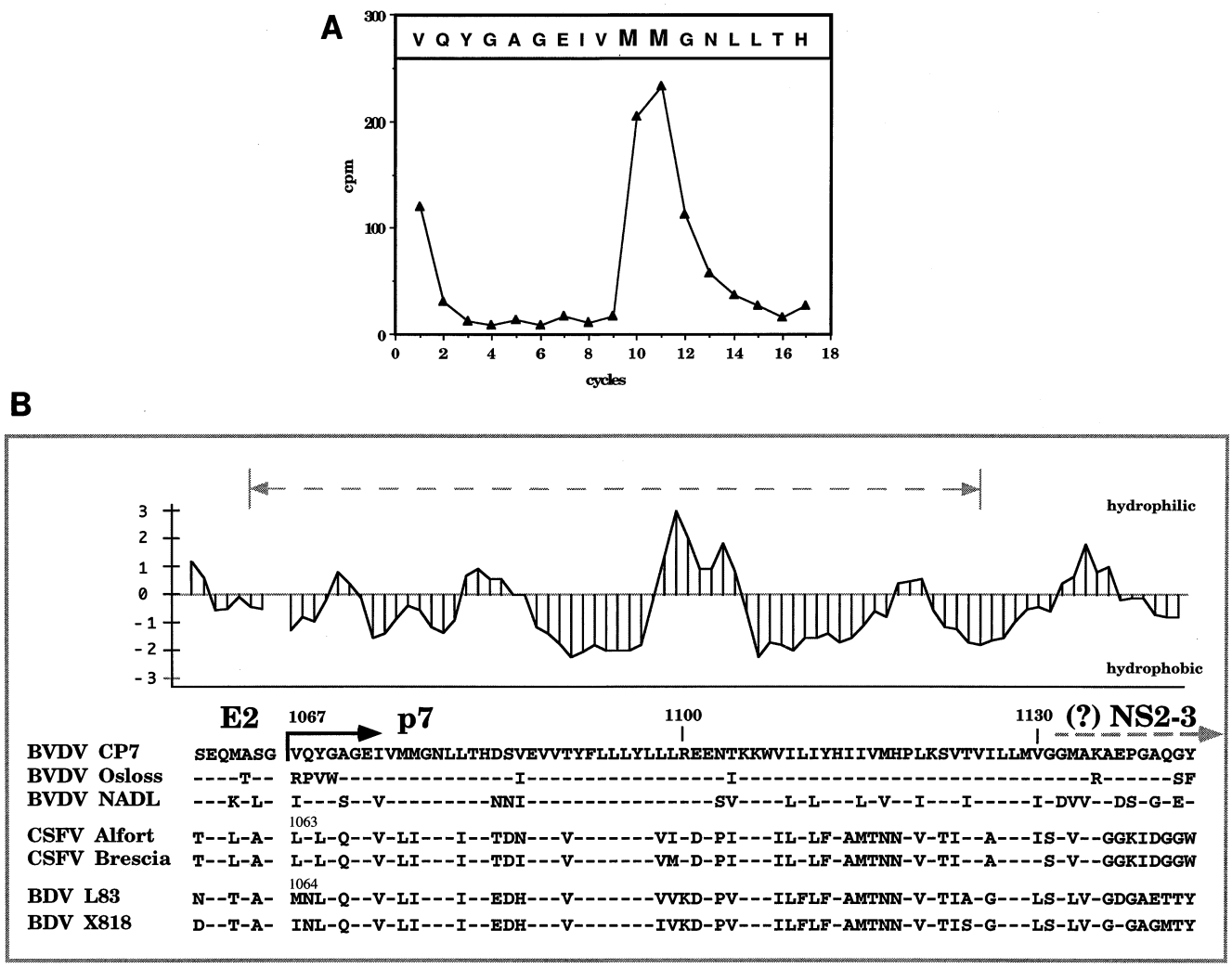

FIG. 6. (A) N-terminal sequencing of p7. In vitro-synthesized, [^{35}S]methionine-labeled p7 was purified by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to peptide sequencing. The graph depicts the distribution of radioactivity released during automated Edman degradation after subtraction of background radiation. At the top of the panel, the amino acid sequence deduced from the BVDV genome is aligned with the appearance of two consecutive methionines, which are unique within the sequence of the in vitro-translated protein. According to this alignment, Val-1067 forms the N terminus of p7. (B) Alignment of the p7 peptide sequences of BVDV CP7 with other pestiviruses. With an N terminus at Val-1067 and a C terminus tentatively located around amino acid 1135, p7 consists of about 70 mostly hydrophobic amino acids. The sequences N terminal to p7 conform to the requirements for signalase ($-1,-3$ rule), and the alignment makes cleavage at the analogous position likely for all pestiviruses. The difference in numbering results from the additional amino acids of the BVDV isolates compared with the CSFV and BDV polyproteins. The overall structure, shown as a Hopps and Wood diagram, is remarkably conserved among the listed strains. The dashed line at the top depicts the amino acid sequence which was fused to the MS2 polymerase and subsequently served for immunization of rabbits.

p7 and NS2 apparently depends on translocation and is facilitated by microsome-associated proteases, presumably signalase. The basic requirements for signalase cleavage are fulfilled in the sequence MASG/V-1067 together with the preceding hydrophobic amino acids (Fig. 6B). Further support for signalase cleavage was obtained by substitution of Arg at the assumed P1 position (Gly-1066). While cleavage between E2p7 and NS2 was unaffected, the generation of E2 and p7 could no longer be detected (Fig. 5, lane 3).

The N terminus of p7 is Val-1067. Further characterization of p7 biosynthesis was expected from N-terminal sequencing of p7. For this procedure, [^{35}S]methionine-labeled p7 was synthesized by in vitro translation, purified by SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (14, 15). The release of radioactivity in the course of the cyclic Edman degradation peaked in cycles 10 and 11, which, according to the positions of the methionine residues, results in Val-1067 being the N terminus of p7 (Fig. 6A). Labeling with a single amino acid was found to be sufficient for radiosequencing because two consecutive methionines are unique within the in vitro-translated protein. p7 sequences of different pestiviruses, including BVDV and CSFV, were aligned (Fig. 6B). It is likely that the p7 proteins of all these viruses share the same N terminus, especially since the signalase cleavage site is preserved.

p7 forms the junction between the structural and the nonstructural genes in pestiviruses, and the same is probably true for hepatitis C virus. The nature of p7 and E2p7 and the peculiar processing of the E2p7NS2 region are well conserved among pestiviruses and hepatitis C virus, which is indicative of a common function for these proteins. The spatial proximity of E2 and p7 in the polyprotein and the formation of an E2p7 fusion protein suggest a role in glycoprotein maturation and/or virus morphogenesis. Pestiviruses are believed to mature at

internal membranes, presumably the endoplasmic reticulum, but distinct steps of pestivirus morphogenesis have not been revealed. Assembly of a number of enveloped viruses requires specific interactions between the membrane-associated glycoprotein(s) and the capsid protein(s) in the cytosol. In general, this is facilitated by the cytoplasmic domain of a glycoprotein which is anchored in the membrane by a transmembrane sequence. A cytoplasmic domain could not be identified for any of the E proteins of pestiviruses. Interestingly, the pestivirus p7 protein contains the charged sequence 1100-REENTKK-1106 (RDEPIKK in CSFV), which probably faces the cytosol (Fig. 6B). It is conceivable that this charged stretch interacts with the capsid protein and initiates the budding process without necessarily being enriched in the virion.

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