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Pestivirus Gene Expression: the First Protein Product of the Bovine Viral Diarrhea Virus Large Open Reading Frame, p20, Possesses Proteolytic Activity

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The positive-strand RNA genome of pestiviruses contains a single large open reading frame (ORF) extending its entire length and is capable of encoding 450 kDa of protein. Studies have been undertaken with the purpose of elucidating the specific mechanisms involved in the biogenesis of the complete complement of pestivirus proteins. Here, we report on gene expression at the 5′ end of the genome of the prototype pestivirus, bovine viral diarrhea virus (BVDV). We demonstrate, using both a cell-free transcription-translation system and a mammalian-cell transient-expression system, that the first protein product of the large ORF of BVDV, the p20 protein, possesses a specific proteolytic activity. The p20 proteinase activity acts to release the p20 protein from the nascent polyprotein. The p20 proteinase activity is not, however, required for downstream glycoprotein processing, indicating translocation of the pestivirus glycoprotein precursor is affected by an internal signal sequence.

Pestiviruses are a group of small, enveloped, positive-strand RNA viruses that represent economically important pathogens of livestock. As a consequence of the recent molecular cloning and nucleotide sequencing of the genomes of several viruses in this group (e.g., bovine viral diarrhea virus [BVDV] [5, 15] and hog cholera virus [12, 13]), our knowledge of the molecular biology of pestiviruses has advanced significantly (6). The pestivirus genome, which is 12 to 13 kb in size, possesses a single large open reading frame (ORF) extending the entire length of the RNA capable of coding for approximately 450 kDa of protein. Studies using sequence-specific immunologic reagents identified virus-encoded protein products encompassing most of the coding potential of the prototype pestivirus, BVDV (4). Those studies led to a preliminary description of the genetic organization of BVDV (4). This first genetic map was recently refined and revised (7). We are now able to account for the entire coding capacity of the BVDV ORF. On the basis of these molecular data and their striking similarities with those of flaviviruses, it has been proposed that pestiviruses be considered a new genus within the family *Flaviviridae* (3).

In previous work investigating the molecular mechanisms of pestivirus protein biogenesis, we described the kinetic progression of precursor polyproteins and processing intermediates to mature viral proteins (7). These investigations suggested at least 10 proteolytic processing sites are involved in the biogenesis of the complete complement of BVDV proteins (Fig. 1A). However, the exact amino acid sequences at which these putative cleavages occur remain to be established. To further understand these suspected processing events, we have endeavored to identify the proteinases affecting these cleavages. Elsewhere, we demonstrate that the pestivirus nonstructural protein p80 is a proteinase involved in polyprotein processing of all viral nonstructural proteins located in the carboxy-terminal two-thirds of the ORF (20). Here, we report on our results involving protein expression at the 5′ end of the BVDV genome.

Small ORFs preceding the large ORF. While the BVDV genome possesses a single large ORF, there are two small ATG-initiated ORFs within the 385 nucleotides preceding the large ORF, one capable of encoding 45 amino acids before terminating and the other comprising 48 amino acids (5). Therefore, to begin our study of gene expression at the 5′ end of the BVDV genome, we constructed two transcription plasmids. In plasmid 5′p20, a segment of the BVDV genome beginning at nucleotide 29 and extending to nucleotide 1110 was engineered downstream of the T7 promoter in a derivative of transcription vector pGem4 (Promega Biotec, Madison, Wis.). This plasmid therefore encompassed both small ORFs as well as the first 244 amino acids of the large ORF (Fig. 1B). Plasmid p20 was engineered similarly, except that the region preceding the large ORF was deleted, allowing the first ATG present downstream of the transcription promoter to be that of the large ORF. When RNA transcripts made from these two plasmids were translated in the presence of [^{3}H]leucine in a reticulocyte lysate system (Promega Biotec), in both cases we observed synthesis of the same single polypeptide, which migrated with an estimated molecular mass of 23 kDa in a 20% polyacrylamide gel (data not shown). No radiolabeled products from the two small ORFs were evident in the 5′p20 translation. The addition of 5′ cap structures to either the 5′p20 or p20 RNA transcript had no qualitative effect and no significant quantitative effect on translation. Apparently, the small ORFs preceding the large ORF in the BVDV genome are not utilized in this translation system. Alternatively, it is possible that products were made but were very rapidly degraded in reticulocyte lysates and therefore were not detectable.

Translation from the 5′ end of the large ORF. The translation product of the p20 RNA transcript was immunoprecipitated with previously characterized antisera directed against

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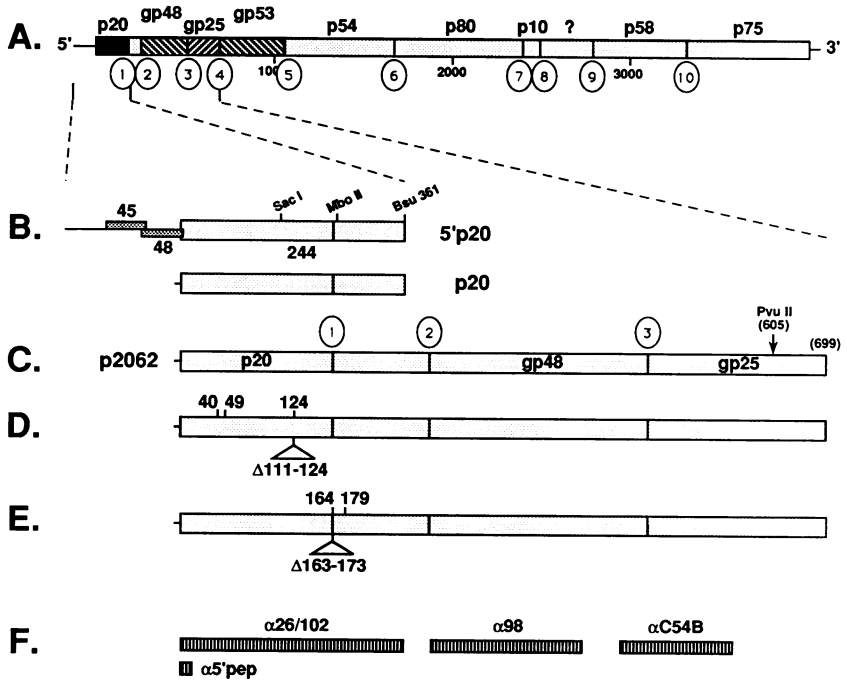

FIG. 1. Schematic representations of the BVDV genome, protein-coding domains, engineered expression constructs, and reagents used in this study. (A) Organization of the BVDV genome, showing the 5′ region preceding the large ORF, the large ORF and approximate coding boundaries of the mature viral proteins (7), and the 3′ noncoding region. The numbers below the ORF box that are not circled indicate amino acid positions within the ORF. The encircled numbers represent putative protein processing sites. (B) Expanded view of the 5′ end of the BVDV genome indicating the BVDV sequences engineered into two transcription vectors downstream of the T7 RNA polymerase promoter. All recombinant plasmids were constructed, used to transform bacteria, and purified by standard methods (11). Plasmid 5′p20 encompasses nucleotides 29 through 1110 of the BVDV sequence and includes the two small ATG-initiated ORFs (45 and 48 amino acids, respectively) which precede the large ORF, as well as a 244-amino-acid-coding segment of the large ORF. The BVDV sequences in plasmid p20 begin at nucleotide 360, just before the ATG of the large ORF at nucleotides 386 to 388, and end at the same position as in vector 5′p20. The positions of three restriction sites are also indicated. (C) Expanded view of the 5′ end of the BVDV genome indicating the BVDV sequences represented in transcription vector p2062. The sequence begins as in vector p20 and extends to nucleotide 2480, encompassing 699 amino acids of the large ORF. The approximate coding domains of proteins p20, gp48, and gp25 are indicated, as are the estimated positions of three polyprotein processing sites. The position of the *Pvu*II restriction site (amino acid position 605) is also indicated. (D) Site-specific mutant derivatives of p2062 used to investigate amino acids critical to proteinase activity. The positions of amino acid changes in four mutant transcription vectors, H40A, H49A, S124A, and Δ111–124, are indicated. (E) Site-specific mutant derivatives of p2062 used to investigate amino acids critical to cleavage site 1. The positions of amino acid changes in three mutant transcription vectors, W164A, K179L, and Δ163–173, are indicated. (F) Antiserum reagents employed in this study. The protein-coding regions encompassed by four immunogens used to generate specific rabbit antisera are indicated.

the authentic viral p20 protein (4). Two antisera were used: one generated against a synthetic peptide representing the first 15 amino acids of the large ORF (α5′pep) and one generated against an engineered recombinant protein encompassing the first 244 amino acids of the ORF (α26/102) (Fig. 1F). The cell-free translation product immunoprecipitated with these antisera was electrophoresed adjacent to p20 protein immunoprecipitated from BVDV-infected cells. The cell-free translation product comigrated with the viral p20 protein with an apparent molecular mass of 20 kDa in this gel system (Fig. 2A).

While it apparently represented the authentic p20 protein, the cell-free translation product appeared to be significantly smaller than the 27 kDa predicted for a product representing the entire p20 transcript ORF (244 amino acids). This could suggest that a proteolytic activity was responsible for producing the observed 20-kDa cell-free product. Alternatively, premature translation termination or anomalous gel migration could explain the apparent size discrepancy. To distinguish among these possibilities, we constructed another transcription vector, designated p2062, in which we extended the BVDV ORF sequence to amino acid 699 so as to include the coding sequences for the downstream viral glycoproteins gp48 and gp25 (Fig. 1C). For RNA synthesis, however, this vector was linearized at a unique *Pvu*II restriction site located at amino acid 605 (Fig. 1C). Translation of the complete ORF in the *Pvu*II-truncated transcripts of p2062 was predicted to yield a 67-kDa polypeptide. However, when the translation products were analyzed by immunoprecipitation with the antisera described above, a 67-kDa product was not obvious. Rather, antiserum α5′pep precipitated only the 20-kDa protein, while antiserum α26/102 recognized a 47-kDa product in addition to p20 (Fig. 2B). The 47-kDa protein was also immunoprecipitated with antisera specific for gp48 (α98) and gp25 (αC54B) (Fig. 1F; not shown in Fig. 2B).

Amino acids important for proteolytic activity are within the p20 coding region. The characteristics of the two translation products of p2062 RNA suggested they represented proteolytic cleavage products of the predicted 67-kDa primary translation product. Either the p20 protein possessed proteolytic activity or there was a specific proteinase present in

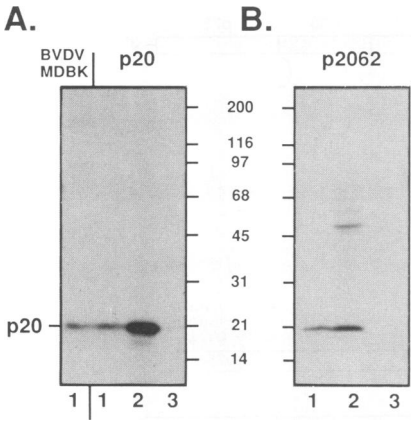

FIG. 2. Immunoprecipitation of translation products from RNA transcripts representing the amino-terminal end of the BVDV large ORF. RNA was synthesized from linear transcription vectors p20 (A) and p2062 (B) by using T7 RNA polymerase according to instructions provided by the manufacturer (Promega Biotec). Reactions were carried out at 40°C for 2 h and terminated by addition of 1 U of RQ1 DNase (Promega Biotec). After 15 min at 37°C, samples were extracted with phenol and chloroform and were ethanol precipitated. All RNA transcripts were determined to be intact and of the expected size by agarose-urea gel electrophoresis (14). Standard translation reaction mixtures (50 μl) contained 1 μg of RNA transcript, 35 μl of rabbit reticulocyte lysate (micrococcal nuclease treated; Promega Biotec), 19 amino acids (20 μM each), and 50 μCi of [^{35}S]methionine (ICN, Costa Mesa, Calif.). Incubations were carried out at 30°C for 1 h. Translation reaction mixtures were prepared for immunoprecipitation as described previously (4), and aliquots of each mixture were incubated with rabbit antiserum α5′pep (tracks 1) or α26/102 (tracks 2) or normal rabbit serum (tracks 3). A lysate of [^{35}S]methionine-labeled BVDV-infected MDBK cells was also immunoprecipitated with α5′pep (A). Radiolabeled, immunoprecipitated polypeptides were resolved on a sodium dodecyl sulfate-containing 10 to 18% gradient polyacrylamide gel and were detected by fluorography. The positions of BVDV protein p20 and the molecular size standards (in kilodaltons) are indicated.

reticulocyte lysates. To investigate the former possibility, we employed site-directed mutagenesis to change amino acids within the p20 coding region to determine the effect on the putative proteinase activity. Upon initiation of this work, we had little basis for selection of residues in p20 to change. Previous computer-assisted analyses of the BVDV sequence had not reported this region of the genome to possess sequence motifs related to known proteinases (1, 9). Our reinspection of this region also failed to reveal compelling relationships. However, a survey of residues at the active centers of viral proteinases indicates that, depending on the enzyme type, four amino acids are generally important: His, Asp, Ser, and Cys (19). In Fig. 3, we indicate the positions within the p20 coding region (up to residue 164) of these residues which are conserved among four pestivirus sequences. We have focused our initial attention on a select few of these.

Mutant transcription plasmids were constructed according to instructions provided with a Muta-Gene mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) by using single-stranded phagemid DNA isolated from transformed *Escherichia coli* CJ236 and mutant synthetic oligonucleotides (18). All mutant constructs were confirmed by DNA sequencing. Two mutant transcription vectors were made in order to address the potential requirement of the histidine residues at positions 40 and 49 for release of p20 from the putative precursor. Translation of mutant RNA H40A (histidine 40 changed to alanine) showed a significant reduction in p20 protein with a concomitant increase in a 67-kDa protein, the size expected for an uncleaved precursor (Fig. 4, track 4). Translation of RNA H49A (histidine 49 changed to alanine) yielded only the precursor polyprotein; absolutely no p20 protein was detected (Fig. 4, track 5). Thus, substitution at either of these histidines affected p20 protein release; however, the change at position 49 appeared most critical. These results are consistent with the view that the p20 protein itself possesses the proteolytic activity observed in these cell-free translation experiments.

That His-49 appeared essential for activity and was located at the amino-terminal end of the polypeptide, as well as amino terminal to all other candidate active-center residues, suggested to us a possible catalytic residue organization of a serine-type proteinase (19), in which a downstream Ser residue might be also important for p20 activity. The Ser at position 124 appeared in the context of a sequence remotely reminiscent of a serine proteinase catalytic center Ser (pestivirus GSDG versus serine proteinase GDSG [1, 9]). However, when we changed the serine codon at position 124 to an alanine codon, translation of the resultant mutant RNA (S124A) still yielded p20, albeit at a slightly reduced level (Fig. 4, track 2). Only a minor amount of the uncleaved 67-kDa precursor polypeptide was observed, indicating that the proteolytic activity of p20 had been affected only slightly by the S-to-A substitution. That the activity was not completely abolished by this change suggests serine 124 is not essential to activity. Interestingly, however, there is a threonine residue very near this serine (Fig. 3; T-122). Whether T-122 is able to partially compensate for the change in S124A was not determined directly. However, we did construct a vector in which 14 amino acids in this region were deleted, including both T-122 and S-124. Translation of this RNA (Δ111–124) resulted in synthesis of only the precursor polypeptide (Fig. 4, track 3).

These initial mutagenesis studies do not serve to establish the proteinase class to which the observed proteolytic activity belongs. Clearly, more extensive efforts in the biochemical characterization of the activity are needed in order to address this issue. However, these results do clearly indicate that the p20 protein is a proteinase and is responsible for its own release from the nascent polyprotein.

Preliminary localization of the p20 proteinase cleavage site. To investigate the site of cleavage by the p20 proteinase (site 1 [Fig. 1]), we first needed to approximate the position of the carboxy terminus of p20. Depending on the polyacrylamide gel system, the p20 protein migrated with an apparent molecular mass of between 19 and 23 kDa. We generated a series of truncated RNA transcripts by cleaving the p2062 transcription vector DNA at conveniently positioned restriction sites (Fig. 1B). RNAs made from these truncated templates were translated, and the resultant runoff polypeptide products were immunoprecipitated and analyzed on a polyacrylamide gel. Truncation at the *Bsu* 361 site provides 244 amino acids within the ORF for translation (calculated molecular mass, 27 kDa); truncation at the *Mbo*II site provides 176 amino acids (calculated molecular mass, 19 kDa); finally, restriction at the *Sac*I site results in 103 translatable amino acids (calculated molecular mass, 11 kDa). Both the *Bsu* 361- and *Mbo*II-truncated RNAs programmed synthesis of an authentically sized p20 protein; the *Sac*I-truncated RNA failed to produce an immunoprecipita-

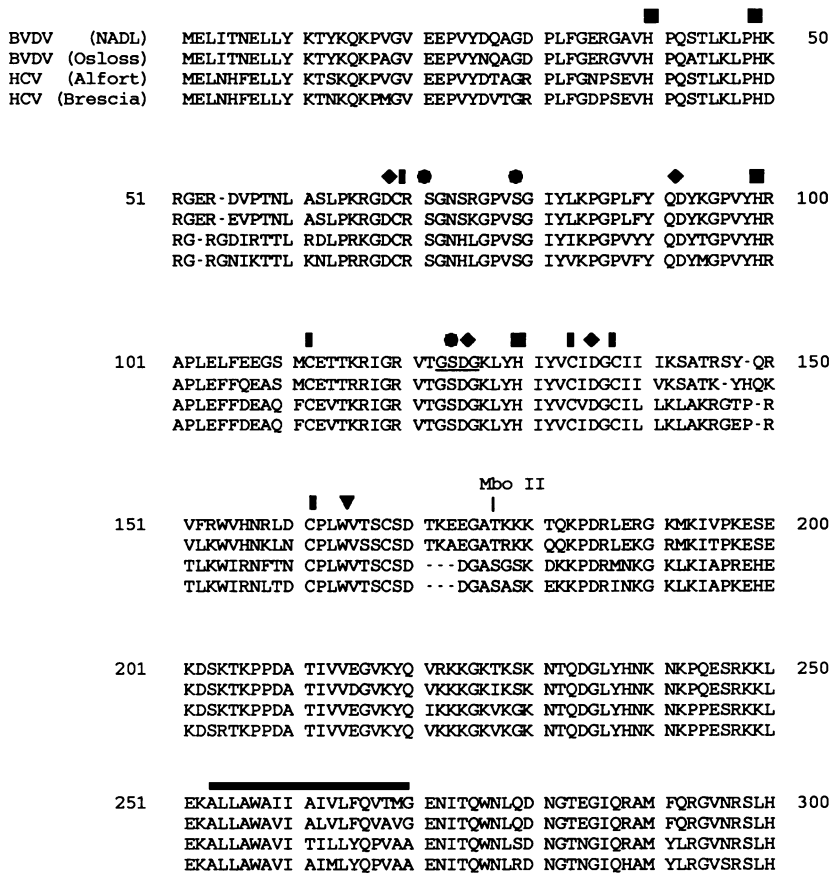

FIG. 3. Amino acid sequence of the amino-terminal end of the pestivirus large ORF. The aligned sequences of the first 300 amino acids of the large ORF of four pestiviruses, two isolates of BVDV (NADL and Osloss) and two isolates of hog cholera virus (HCV) (Alfort and Brescia), are presented. The positions of conserved histidine (■), aspartic acid (◆), serine (●), and cysteine (▮) residues within the first 164 amino acids of the ORF are indicated. Also indicated are the positions of the *Mbo*II restriction site, Trp-164 (▼), and the stretch of hydrophobic amino acids (solid bar) in the pestivirus sequences.

ble product (Fig. 5A). These data indicate the carboxy end of the p20 protein was close to or upstream of the *Mbo*II restriction site.

We then constructed a number of p2062 transcription plasmids in which residues in and around this region were changed. Because of a similarity to the dibasic residues found at many of the cleavage sites of flavivirus polyproteins (2), the central lysine residue at position 179 of a tribasic sequence (Fig. 3) was changed to leucine (K179L). This change had no effect on the release of p20 (Fig. 5B, track 2). Since a glutamic acid-glycine pair appeared in this region and this sequence is recognized as a cleavage site in some picornaviruses (19), the mutant transcript G175V (glycine 175 changed to valine) was constructed. This change also failed to alter the wild-type pattern of translation (data not shown). A transcription vector having a more radical alteration, a deletion of 11 amino acids (positions 163 through 173; vector designated Δ163–173) that retained an ORF was made. Translation of Δ163–173 RNA resulted in synthesis of only a precursor protein of the expected size; no p20 was produced (Fig. 5B, track 3). This result was followed by the generation of several mutants involving substitution of single amino acids within the sequence deleted in construct Δ163–173. Transcripts designated E173L, T171L, and S169A all translated identically to the wild-type transcript, producing p20 and the 47-kDa protein (data not shown). However, a change of the tryptophan at position 164 to alanine (W164A) completely eliminated the appearance of p20 and resulted in production of only the 67-kDa precursor protein (Fig. 5B, track 4). Thus, the tryptophan residue at position 164 is critical to p20 biogenesis. Whether this amino acid represents the actual site of cleavage by the p20 proteinase and the carboxy-terminal amino acid of the p20 protein cannot be established from these data. However, it is interesting that chymotrypsin and the capsid protein autoproteinases of alphaviruses, both serine proteinases, cleave at tryptophan (16, 17). Moreover, that a valine residue occurs at the putative +1 position is consistent with the observation that most N termini created by viral proteinases consist of either Gly, Ser, Ala, Thr, Met, or Val (19).

Gene expression and protein processing at the amino-terminal end of the BVDV large ORF in transfected mammalian cells. The results of our cell-free translation studies indicate the p20 protein is a proteinase and is responsible for its own release from a nascent polyprotein by cleavage at site 1 (Fig. 1). However, within the primary translation product of the p2062 transcript (the 67-kDa polypeptide), two additional polyprotein processing sites are present (sites 2 and 3 [Fig. 1C]). Apparently, in this test system, these sites are not acted on by the p20 proteinase. Sites 2 and 3 are

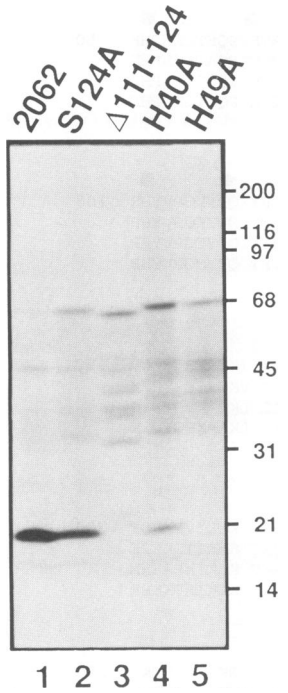

FIG. 4. Immunoprecipitation of translation products from site-specific mutant RNA transcripts of the p2062 vector. RNA products from the indicated transcription vectors were translated in reticulocyte lysates in the presence of [^{35}S]methionine. The reaction mixtures were then immunoprecipitated with antiserum α26/102, and the radiolabeled polypeptides were resolved by electrophoresis in a sodium dodecyl sulfate-containing 10 to 18% gradient polyacrylamide gel. Molecular size standards are indicated in kilodaltons.

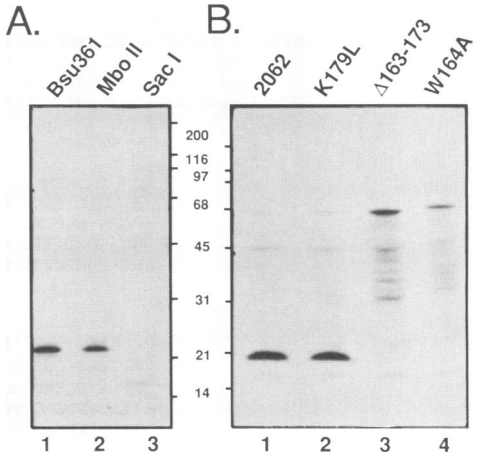

FIG. 5. Localization of the p20 proteinase cleavage site. (A) Transcription vector p2062 was linearized by restriction at either the *Bsu* 361, *Mbo*II, or *Sac*I site (Fig. 1B). The three restricted plasmids were transcribed and translated. The reaction mixtures were then immunoprecipitated with antiserum α26/102, and the radiolabeled polypeptides were resolved by electrophoresis in a sodium dodecyl sulfate (SDS)-containing 10 to 18% gradient polyacrylamide gel. (B) Translation products from RNA transcripts of p2062 and the indicated site-specific mutant vectors were immunoprecipitated with antiserum α26/102. The radiolabeled polypeptides were resolved by electrophoresis in an SDS-containing 10 to 18% gradient polyacrylamide gel. Molecular size standards are indicated in kilodaltons.

located in the region of the BVDV ORF that encodes viral glycoproteins. Since the mature BVDV glycoproteins are produced in vivo by the progressive proteolytic processing of several glycosylated precursor polyproteins (7), cleavage at sites 2 and 3 might be effected by membrane-associated cellular processing enzymes (e.g., signalases) and not by virus-encoded proteinases. However, the in vitro translation of p2062 RNA in the presence of functional microsomes produced a protein product profile unchanged from that observed in the absence of microsomal membranes (data not shown).

Therefore, to further investigate gene expression of this region of the BVDV genome and the role the p20 proteinase might play in vivo, we employed a mammalian-cell transient-expression system (8). The intact transcription plasmid p2062 (10 μg) was transfected into BHK cells infected 30 min earlier with 5 PFU of the T7 RNA polymerase-expressing vaccinia virus vTF7-3 per cell by using a Cellphect kit (Pharmacia, Piscataway, N.J.). Three hours later, the cells were radiolabeled with [^{35}S]methionine for 2 h, after which a cleared cell lysate was prepared for immunoprecipitation analysis. Antiserum α26/102 (Fig. 6, track 3), as well as α5′pep (data not shown), immunoprecipitated the p20 protein. Antiserum α26/102, but not α5′pep (not shown), also specifically recognized proteins of 78 and 52 kDa (track 3). Use of pooled antisera α98 and αC54B revealed proteins of 62 and 25 kDa clearly, as well as a less intense polypeptide of 48 kDa (track 4). All proteins immunoprecipitated from the transfected cells comigrated with authentic viral proteins immunoprecipitated from a BVDV-infected MDBK cell lysate run in adjacent tracks (tracks 1 and 2).

To demonstrate that the transiently expressed polypeptides comigrating with the viral glycoproteins were indeed glycosylated, we subjected a portion of the immunoprecipitates to endoglycosidase treatment (tracks 5 and 6). Protein p20 was unaffected by this treatment, while all of the presumptive viral glycoproteins were converted to the previously described sizes of unglycosylated authentic BVDV glycoproteins (4): gp62 to 42 kDa, gp48 to 27 kDa, and gp25 to 20 kDa. Endoglycosidase treatment of the α26/102 immunoprecipitate resulted in the disappearance of the diffuse gp78 and the appearance of a similarly diffuse band at approximately 52 kDa as well as a new band at 39 kDa (track 5). Because of the distinct character of the radiolabeled 52-kDa bands before and after endoglycosidase treatment, it appears gp78 was degglycosylated to a polypeptide migrating with an apparent molecular mass of 52 kDa (representing protein sequences from site 1 to the end of the construct ORF [Fig. 1C]) and the original 52-kDa glycoprotein was itself converted to the 39-kDa band (encompassing protein sequences from site 1 to site 3 [Fig. 1C]). This interpretation is consistent with previously described positioning of the gp78 coding region (7). Thus, the expression of the BVDV ORF present in plasmid p2062 in this in vivo transient expression system faithfully produced the p20 protein, the previously described glycoprotein precursor polypeptides gp78 and gp62, and the mature glycoproteins gp48 and gp25.

To determine the requirement of a functional p20 proteinase for these processing events, we next analyzed BVDV protein expression in this system by the p20 proteinase-negative derivative of p2062, H49A. Antiserum α26/102 immunoprecipitated polypeptides of approximately 110, 77, and 34 kDa (Fig. 6, track 7). These same polypeptides were also recognized by antiserum α5′pep (not shown). Abso-

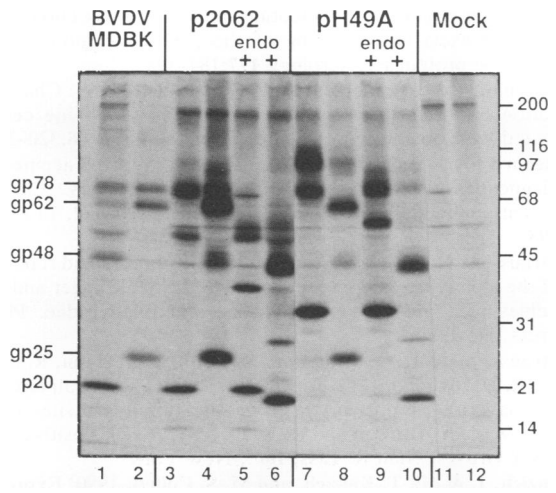

FIG. 6. Transient expression of constructs p2062 and pH49A in mammalian cells. Transcription vector DNAs p2062 and pH49A were individually transfected into vaccinia virus vTF7-3-infected BHK cells. After being radiolabeled with [^{35}S]methionine, cells were harvested, and cleared cell lysates were prepared. Aliquots of these lysates were immunoprecipitated with antiserum α26/102 (tracks 3 and 7) or a pooled combination of antisera α98 and αC54B (tracks 4 and 8). Mock-transfected cell lysates were similarly prepared and immunoprecipitated with antiserum α26/102 (track 11) or the combination of antisera α98 and αC54B (track 12). Portions of the p2062 and pH49A immunoprecipitates were further subjected to endoglycosidase treatment (endo +) as previously described (10). Tracks 5 and 9, endoglycosidase-treated α26/102 immunoprecipitates; tracks 6 and 10, endoglycosidase-treated α98 and αC54B combination immunoprecipitates. To serve as markers, authentic BVDV proteins immunoprecipitated from virus-infected MDBK cell lysates were run in adjacent tracks (track 1, combined α26/102 and α98 immunoprecipitate; track 2, αC54B immunoprecipitate). Radiolabeled polypeptides were resolved by electrophoresis in a sodium dodecyl sulfate-containing 10 to 18% gradient polyacrylamide gel and detected by fluorography. Authentic BVDV proteins are indicated at the left, and molecular size standards are indicated at the right in kilodaltons.

lutely no p20 protein was detectable in these cells. Immunoprecipitation with pooled antisera α98 and αC54B identified polypeptides that comigrated with the viral glycoproteins gp62, gp48, and gp25, as well as a minor amount of a 110-kDa protein (track 8). Endoglycosidase treatment of these immunoprecipitates failed to alter the migration of the 34-kDa protein (track 9) but did convert the 110-kDa protein to 78 kDa (track 9), the 77-kDa protein to 58 kDa (track 9), and gp62, gp48, and gp25 to their expected unglycosylated sizes (track 10).

Taking into account the molecular mass estimates of the observed polypeptides and their antibody reactivities, these data may be interpreted as follows. In H49A-transfected cells, the p20 proteinase is inactive; cleavage at site 1 does not occur and the p20 protein is not released from its precursor polyprotein. However, in this total absence of p20 proteinase activity, proteolytic processing of the polyprotein does occur at sites 2 and 3 (Fig. 1C). The 34-kDa protein represents the coding region from the beginning of the ORF to site 2. The glycosylated protein of 77 kDa encompasses the region from the ORF ATG to site 3. Both of these polypeptides represent partially processed products of the ORF. On the other hand, the 110-kDa glycoprotein represents the totally uncleaved polyprotein of the entire ORF. Finally, complete processing, and apparently normal glycoprotein glycosylation, does take place, as evidenced by the production of the mature glycoproteins gp48 and gp25. Apparently, the p20 proteinase activity, while essential for its own release from the nascent polyprotein, is not required, nor is it a prerequisite, for glycoprotein biogenesis.

The proteolytic cleavages at sites 2 and 3 are likely catalyzed by host cell proteinases, presumably signalases or Golgi complex-associated enzymes. However, for these cleavages and protein glycosylation to occur, the glycoprotein precursor polypeptide must be translocated into the endoplasmic reticulum. The p20 protein itself lacks an amino-terminal membrane insertion or signal sequence. This, coupled with the observation that p20 need not be released from the nascent polyprotein before membrane transit, suggests that translocation into the endoplasmic reticulum of the pestivirus glycoprotein precursor is affected by an internal signal sequence. The carboxy-terminal region of p20 does not contain a significant hydrophobic domain (Fig. 3). Furthermore, the newly formed amino terminus is quite hydrophilic and would not be predicted to act as a membrane insertion sequence. However, there does exist a strongly hydrophobic stretch of 18 amino acids (residues 253 to 270 [Fig. 3]) positioned very near, and likely just amino-terminal to, our estimated location of proteolytic cleavage site 2. This sequence, some 90 amino acids downstream of the position of p20 proteinase cleavage (site 1), may represent the internal signal sequence used for translocation of the nascent pestivirus glycoprotein precursor polypeptide into the endoplasmic reticulum.

Since the p20 protein possesses proteolytic activity and is also likely the viral capsid protein, similarities to the alphavirus capsid autoproteinases are obvious. However, the apparent features of glycoprotein processing appear quite different from those of alphaviruses (16). Rather, they are reminiscent of mechanisms thought to be employed by flaviviruses (2). Further studies of pestivirus gene expression and the role of p20 and its associated proteinase activity are clearly in order. Among the issues that require additional attention are establishment of the proteinase class to which p20 belongs, determination of its cleavage site specificity, and description of its ability to act either intramolecularly (in *cis*) or in *trans*. The work reported here represents initial investigations in the first two areas. Regarding the last area, in work not presented here, we have been unable to demonstrate, in either the cell-free translation or the mammalian-cell transient-expression system, a *trans* activity associated with the p20 proteinase. While we suspect the p20 proteinase acts in *cis*, additional studies are necessary to establish this.

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