Baculovirus expression of pestivirus non-structural proteins

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Bovine viral diarrhoea virus (BVDV) belongs to the pestivirus group, a genus within the Flaviviridae family. It possesses a positive-sense ssRNA genome with a single large open reading frame (ORF) encoding about 4000 amino acids. Here we report the continuation of our studies of pestivirus protein biogenesis, involving expression from the viral non-structural protein-encoding region. The 3'-terminal 60% of the BVDV ORF was cloned into a plasmid transfer vector which was then used to construct a recombinant baculovirus. Infection of Spodoptera frugiperda Sf9 cells with this recombinant virus resulted in the production of the expected mature viral proteins. Polyprotein processing by the BVDV p80 proteinase appeared to be nearly identical to that observed in authentic BVDV-infected bovine cells, and as previously shown to occur when expression of the same region was studied in a mammalian cell transient expression system. However, one viral proteolytic cleavage did not occur in the baculovirus-infected insect cells; the viral p80 proteinase failed to act at its own N terminus. This recombinant baculovirus/insect cell expression system provides an abundant source of BVDV non-structural proteins. Therefore we explored the utility of the proteins produced in this system for the detection of anti-BVDV antibodies in bovine sera. In preliminary experiments using these antigens in an ELISA we found a positive correlation between the presence of ELISA-reactive antibody and virus-neutralizing activity.

Pestiviruses are a group of enveloped RNA viruses causing economically important diseases of cattle, pigs and sheep world-wide. The pestivirus genome is a positive-sense ssRNA of about 12 to 13 kb and consists of a single large open reading frame (ORF) encoding approximately 4000 amino acids (Collett et al., 1989; Collett, 1992). However larger genomic sizes have recently been observed for some bovine viral diarrhoea virus (BVDV) isolates (Meyers et al., 1991). The complete complement of pestivirus-encoded proteins has been largely elucidated (Collett et al., 1988a, 1991; Thiel et al., 1991; Collett, 1992) and, whereas the recently identified viral capsid protein (p14) and the three envelope glycoproteins are encoded near the 5' terminus of this ORF (Fig. 1a), the very first product of the ORF (p20) does not appear to be associated with virions (Thiel et al., 1991). The major viral non-structural polypeptides are encoded in the 3'-terminal 75% of the ORF (Fig. 1a).

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Our interest has been in gaining an understanding of pestivirus protein biogenesis and protein function. In previous work using both a cell-free transcription-translation system and a mammalian cell transient expression system, we have shown that the p20 protein possesses proteolytic activity, and that this activity is responsible for cleavage at site 1 (Fig. 1a) and for p20 protein release from the nascent polyprotein (Wiskerchen et al., 1991). We have also shown that in the mammalian cell transient expression system the p80 protein of BVDV is the serine proteinase required for the proteolytic processing of the viral non-structural proteins, cleaving at sites 6, 7, 8, 9 and 10 (Fig. 1a; Wiskerchen & Collett, 1991). Here, we have investigated the use of a baculovirus/insect cell expression system for the study of pestivirus protein biogenesis and for the production of pestivirus polypeptides. Specifically, we constructed a recombinant baculovirus containing the 3'-terminal 60% of the BVDV ORF encoding the non-structural proteins. We describe pestivirus gene expression by this recombinant virus and the possible use of the BVDV antigens produced in this system.
Baculovirus transfer plasmid pVLD2-133 was assembled in two stages, employing standard recombinant DNA procedures (Maniatis et al., 1982). First, intermediate vector pVLD2-42 was constructed. A BgIII–BspE1 restriction fragment was excised from BVDV transcription plasmid D2-3' (Wiskerchen & Collett, 1991). The BgIII site is vector-derived and is positioned immediately upstream of BVDV nucleotide (nt) 5128 (BVDV nt coordinates are according to the sequence of the BVDV NADL isolate; Collett et al., 1988b); the BspE1 site is at BVDV nt 8705. This site was made blunt using the Klenow fragment of DNA polymerase I and nuclease triphosphate incubation prior to BgIII restriction. The resulting 3580 bp fragment was ligated to BamHI- and Smal-digested pVL1393 transfer vector DNA (Luckow & Summers, 1989) to create plasmid pVLD2-42. pVLD2-42 was then digested with NcoI (site at BVDV nt 8317) and Xbal (downstream vector site). The Xbal site was filled using the Klenow fragment of DNA polymerase I. The BVDV sequence-containing plasmid was ligated to a BVDV gene fragment derived from transcription plasmid D2-3' using NcoI (site at BVDV nt 8317) and PvuII (site at BVDV nt 12348) restriction, yielding the recombinant transfer vector pVLD2-133. Plasmid pVLD2-133 DNA was then co-transfected with wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) genomic DNA into Spodoptera frugiperda SF9 cells by standard procedures (Summers & Smith, 1987). Recombinant virus was identified by dot hybridization with 32P-labelled BVDV DNA probes and was purified by three cycles of limiting dilution cloning. Cells infected with the resultant virus, designated bacD2-133, were polyhedrin-negative.

The portion of the BVDV ORF engineered behind the baculovirus polyhedrin promoter in bacD2-133 is schematically shown in Fig. 1(b). The first translation start site downstream of the polyhedrin promoter occurs at the methionine codon at position 1591 in the BVDV ORF, estimated to be about 190 nucleotides upstream of the p80 coding region. The ORF then continues to the last codon of the authentic BVDV ORF at nt 11964, at which point the natural termination codon is encountered.

The predicted primary translation product of the BVDV ORF in bacD2-133 is calculated to have an M, of 264K. BVDV-specific polypeptide synthesis by recombinant virus bacD2-133 was investigated by infecting SF9 cells at a multiplicity of 5, and then incubating the culture in methionine-free Grace’s medium (Gibco) for 1 h at 40 h post-infection. The culture was then radio-labelled for 5 to 6 h in methionine-free medium supplemented with 100 μCi of Trans 35S-label (ICN) per ml. Cells were harvested and prepared for immunoprecipitation in SDS lysis buffer (Collett et al., 1988a). Aliquots were immunoprecipitated with one of several specific antisera. These antisera, schematically shown in Fig. 1(c), collectively are capable of detecting all previously described polypeptide products encoded by the region of the BVDV ORF under study (Collett et al., 1991).

Fig. 2 shows the results of this immunoprecipitation analysis. Antisera a1770 and aB10 immunoprecipitated a major product of about 87K (lanes 2 and 3) which migrated significantly more slowly than the authentic p80 protein from BVDV-infected bovine cells (lane 1). Antiserum aB10 also recognized specific polypeptide bands of 42K and 10K (lane 3), corresponding to a normally short-lived precursor polypeptide, p42, and the p10 protein found in BVDV-infected cells (lane 1; Collett et al., 1991). Although not shown in Fig. 2, these same two polypeptides were also immunoprecipitated by antiserum a2396 (Fig. 1c), whereas the 87K protein was not recognized by this antiserum. Finally, antiserum aZ2D and aB3B recognized 58K and 75K proteins, respectively (lanes 4 and 5), each comigrating with authentic viral p58 and p75 (lane 6).

In this insect cell expression system, the BVDV sequences contained within the bacD2-133 virus were clearly expressed and processed. The predicted 264K primary translation product was not observed. Rather, we were able to detect protein products indicative of proteolytic processing at all the expected sites, with one exception. This exception was site 6 (Fig. 1a); there was no indication of cleavage at this site. The 87K product precipitated with antiserum a1770 and aB10, but not by a2396, is of the size and immunoreactivity expected for a protein beginning at the BVDV translation start site in bacD2-133 and ending at site 7. Expression of this same
The p80 proteinase in this system is actually an active viral protein. p58 and p75, a normal precursor, are efficient at sites 9 and 10. We were able to detect only a trace amount of the 133K polyprotein precursor to the p80 proteinase and its substrates are active and seen in BVDV-infected cells (p133, Fig. 2, lane 6). That the p80 proteinase in this system is actually an N-terminal-extended version (p87) indicates that this enzyme can tolerate such additional sequences and does not require the putative cleavage at site 6 for activity. This observation further supports the suggestion that the pestivirus p125 protein found in bovine cells infected with non-cytopathic (ncp) isolates of BVDV is an active proteinase (Wiskerchen & Collett, 1991).

Future investigations of pestivirus protein expression and processing in this system, including pulse-chase kinetic and polypeptide stability studies, should lead to a clearer picture of the sequence of events in viral non-structural protein biogenesis. This bioproduction system should also provide an abundant source of the mature BVDV non-structural proteins for detailed structural and functional studies. Although we have not yet determined the absolute amount of the BVDV proteins expressed in bacD2-133-infected Sf9 cells, the 87K and p75 proteins can be readily detected in Coomassie blue-stained gels after immunoprecipitation. Work is under way toward the biochemical purification of the BVDV polypeptides produced in this system.

One practical application for this expression system may lie in its use for serodiagnosis of pestivirus infections. The p80 protein is highly conserved among pestiviruses (Collett et al., 1989; Meyers et al., 1989; Moormann et al., 1990) and is an immunodominant antigen, convalescent bovine sera containing high levels of antibodies to p80 (Donis & Dubovi, 1987). Given the level of expression of this antigen and its antigenicity, we have in preliminary work investigated the utility of bacD2-133 antigens produced in Sf9 cells for the detection of anti-BVDV antibodies in bovine sera. We explored the use of a simple ELISA format for this purpose. Antigen extracts were prepared from bacD2-133-infected [positive (P)] and wild-type AcNPV-infected [negative (N)] cells as follows. PBS-washed frozen cell pellets (2 × 10^7 virus-infected cells) were resuspended in 5 ml of 2% Triton X-100, 10 mM-glycine, 3.8 mM-Tris base (final pH 8.8). After sonication, the solution was cleared at 10 000 g for 30 min, and the supernatant was divided into aliquots and stored at −70°C. Microtitre plates (96-well, Immulon II, Dynatech) were coated overnight at 4°C with 50 μl P or N antigen diluted 1:200 in 60 mM-carbonate buffer pH 9.6. After washing with PBS, 0.1% Tween 20 and blocking for 60 min at 37°C with PBS, 0.1% Tween 20, 0.1% gelatin (PBSTG), bovine sera (50 μl) serially diluted in PBSTG were added and incubated at 37°C for 60 min. After three washes with PBSTG, a 50 μl volume of horseradish peroxidase-conjugated Protein G (1:800 dilution; Zymed) was applied for 30 min at 37°C. After further washing, a 100 μl volume of ABTS substrate (Kirkegaard & Perry) was added, and absorbance readings at 405 nm were determined after 30 min at room temperature.
Fig. 3. Evaluation of bovine sera by recombinant baculovirus antigen-down ELISA. Sera were serially diluted in PBSTG from 1:100 to 1:12800. Fifty microlitres of each dilution was placed into paired microtitre plate wells previously coated with either bacD2-133 antigen (P) or control AcNPV antigen (N) as described in the text. The averaged P:N absorbance values (A<sub>405</sub>) for each dilution series were then plotted. In (a) to (d) respectively, serum samples were (C) 1, 7, 13 and 19, (■) 2, 8, 14 and 20, (△) 3, 9, 15 and 21, (▲) 4, 10, 16 and 22, (○) 5, 11, 17 and 49, and (●) 6, 12, 18 and NBS.

Table 1. Comparison of VN activity in bovine sera and antibody detection by a baculovirus-produced antigen-down ELISA

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* Those samples for which the results of the VN assay and ELISA differed.

A panel of 22 bovine sera, in addition to one known positive (no. 49) and one known negative (NBS), was evaluated in this ELISA. This serum panel represents random field samples submitted for analysis to the Diagnostic Laboratory, New York State College of Veterinary Medicine at Cornell University, New York, U.S.A. Serial serum dilutions were assayed. The ELISA results are shown in Fig. 3, plotted as P:N values for each dilution series. From these data, endpoint ELISA titres were determined for each serum sample. These titres were defined as the reciprocal of the serum dilution P:N value that was three times the mean no antigen-down value, and are shown in Table 1.

This same panel of bovine sera was then evaluated for virus-neutralizing activity. Virus neutralization (VN) was determined as previously described using the Singer isolate of BVDV (Donis & Dubovi, 1987). VN titres represent the reciprocal of the highest serum dilution that completely prevented cytopathic changes in the cell cultures. Table 1 shows a comparison of the ELISA endpoint titres with the VN data. Of the 24 sera analysed, nine were negative by both VN and ELISA. Of the 15 positive for VN, 11 scored positive in the ELISA. Thus, with this small sample number (n 24), the concordance between VN and the recombinant baculovirus antigen ELISA was 83% (Spearman rank, 0.797; t = 6.19, P < 0.001).

Three of the four VN-positive, ELISA-negative sera exhibited VN activity of 1:16 or less (no. 2, no. 11 and no. 21), suggesting the sensitivity of VN might exceed that of the ELISA. However, the fourth serum sample (no. 16) showed a higher VN titre (1:96), yet scored negative in the ELISA, suggesting there may be other
explanations for the disagreement between the results of the ELISA and the VN assay. One possibility is that in some cases the observed neutralization may not be associated with IgG binding to viral antigens. This suggestion is supported by our observation that all four of these sera failed to immunoprecipitate radiolabelled BVDV (NADL) polypeptides (data not shown).

Another consideration for samples showing differing results in this ELISA and a VN assay involves the fact that these two assays actually measure distinct specificities of anti-BVDV antibodies. The VN assay is probably measuring antibodies against the viral structural glycoproteins only. Of the many monoclonal antibodies recognizing pestivirus antigens, VN activity has been found only for those reactive with the envelope glycoproteins (Collett et al., 1989). The present ELISA involves detection of antibodies to viral non-structural proteins which, in cattle, are directed principally against the p80 (87K) antigen (Donis & Dubovi, 1987; Collett et al., 1988a). There is currently no evidence that antibodies to the p80 protein are capable of VN.

Finally, differences in the present study may reflect the fact that the ELISA antigens were derived from one BVDV isolate (NADL), and the VN assay employed another (Singer strain). In considering assays for the detection of antibodies to BVDV in a veterinary setting, field isolate variation represents a formidable problem. Using nucleic acid hybridization probes representing several regions of the BVDV genome, Kwang et al. (1991) have shown a p80 region probe to have the highest detection rate (96% of cytopathic (cp) and 43% of ncp viruses) with a panel of field isolates. Two probes representing the glycoprotein region had significantly lower detection rates (75 to 79% for cp and 20 to 24% for ncp viruses). These results suggest that the p80 protein may offer a more conserved antigen for antibody detection in the field. Therefore, our preliminary results with the bacD2-133 antigen ELISA indicate further effort in exploiting this system for screening of diagnostic specimens is warranted. We plan to develop it more fully, exploring modifications that may increase assay sensitivity. Additionally, we are in the process of evaluating this test on a much larger scale so as to determine its ultimate usefulness.

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References


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