Purification and characterization of the major group-specific core antigen VP7 of bluetongue virus synthesized by a recombinant baculovirus

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The major core protein, VP7, of bluetongue virus serotype 10 (BTV-10) has been purified from insect cells infected with a genetically manipulated recombinant baculovirus. The high level expression of VP7 (in excess of 100 mg per litre of culture) and its presence in the soluble fraction of infected cells following lysis by detergent has allowed the purification of the protein virtually to homogeneity (95%) by a simple two-step procedure of ammonium sulphate fractionation and ion-exchange chromatography. The purified antigen is highly immunogenic and has been shown in an ELISA to be reactive with antisera of 24 BTV serotypes (1 to 24) as well as with an antiserum raised to African horsesickness virus type 4 (AHSV-4), a representative of another serogroup of orbiviruses. In confirmation of these data a monospecific antiserum raised with the expressed product has been shown by Western blot analyses to react with other BTV serotypes as well as with two serotypes of epizootic haemorrhagic disease virus (EHDV-1 and EHDV-2), a closely related orbivirus. The data indicated that VP7 is a highly conserved protein amongst BTV serotypes and at least partly conserved amongst three serogroups of orbiviruses.

Introduction

Bluetongue virus (BTV), an arbovirus belonging to the Orbivirus genus of the family Reoviridae, infects sheep and cattle in many parts of the world. Twenty-four distinct serotypes of the virus have been recognized. The virus is spread by Culicoides vectors. Like other members of the Reoviridae family, BTV has a double-capsid structure, with the inner shell exhibiting icosahedral symmetry. Two proteins (namely VP2 and VP5) form the outer capsid. They are loosely bound to the inner capsid. This results in an ill-defined outer structure (Verwoerd et al., 1972). Recent three-dimensional structural analyses of the inner capsid have indicated that it is 69 nm in diameter and divided into two concentric shells enclosing the innermost core (B. V. V. Prasad, personal communication). The first shell, i.e. the outer component of the inner capsid, consists of the major protein VP7. This protein appears to be clustered into trimers at all the local and strict three-fold axes (B. V. V. Prasad, personal communication). This shell is built on a second shell which is composed of the VP3 protein. These two shells enclose the remaining three minor proteins of the innermost core, VP1, VP4 and VP6, and the 10 dsRNA segments of the genome. In addition to the seven structural proteins (VP1 to VP7), three non-structural, virus-encoded proteins (NS1 to NS3) are synthesized in BTV-infected cells (Huismans, 1979).

The outer capsid protein, VP2, elicits the production of neutralizing antibodies in animals and has type-specific epitopes (Huismans et al., 1987a; Roy et al., 1990b). Among other BTV proteins, VP7 has been shown to be a group-specific antigen (Huismans & Erasmus, 1981; Gumm & Newman, 1982) and is therefore a candidate for a diagnostic reagent. VP7 obtained from cells infected with BTV has been employed as such a reagent (Gumm & Newman, 1982).

Recombinant baculovirus vectors such as those constructed from Autographa californica nuclear polyhedrosis virus (AcNPV) have been used to synthesize a number of eukaryotic proteins (Luckow & Summers, 1988). Foreign genes are expressed in insect cells under the control of the strong polyhedrin promoter of AcNPV (Smith et al., 1983; Matsuura et al., 1987). Previously, we have described high yields of a number of BTV proteins using such vectors. In this paper we describe the construction and isolation of a recombinant baculovirus that synthesizes the VP7 of BTV-10 in infected Spodoptera frugiperda cells. The biologically active form of the expressed VP7 protein is produced in excess of 100 mg per litre of culture. A simple procedure of purification has been derived and the cross-reactivity of
the purified protein with all 24 BTV serotypes has been demonstrated. In addition, we present data which indicate a relationship between BTV and African horse-sickness virus (AHSV).

**Methods**

**Virus and cells.** *S. frugiperda* cells were grown in suspension or monolayer cultures at 28°C in TC100 medium supplemented with 10% foetal calf serum. AcNPV and recombinant baculoviruses were plaque-purified and propagated as described by Brown & Faulkner (1977). BTV and two serotypes of epizootic haemorrhagic disease virus (EHDV) were grown on monolayers of BHK cells in Eagle's medium containing 3% foetal calf serum and the virus particles were purified using methods described by Mertens et al. (1987).

**DNA manipulations and recombinant plasmid construction.** Standard DNA manipulation techniques were used to construct recombinant plasmids (Maniatis et al., 1982). Restriction enzymes, T4 DNA ligase and the Klenow large fragment of DNA polymerase I were purchased from Boehringer Mannheim.

The construction of plasmid pAcYM1 BTV-10.7 is summarized in Fig. 1. The full-length cDNA of segment 7 (S7) of BTV RNA had previously been cloned into the *PstI* site of pBR322 (Yu et al., 1988). Since the S7 sequence contains an internal *PstI* site the gene was excised from pBR322 by partial digestion with *PstI*, and recloned into the symmetric polylinker of plasmid pUC4K (Fig. 1). The S7 DNA was isolated by *EcoRI* digestion and the homopolymeric tails which had been added in the original cloning procedure were removed by digestion with *Bal31*. The products were then repaired using the Klenow fragment of DNA polymerase I and inserted into pUC4K which had been digested previously with *SalI*, repaired and dephosphorylated as shown in Fig. 1. After transformation of *Escherichia coli* and selection of drug-resistant colonies, clones containing the S7 DNA were identified by colony hybridization (Grunstein & Hogness, 1975). These were subjected to restriction enzyme analysis to identify clones possessing an insert of the length likely to contain the complete coding sequence of S7 without the homopolymeric sequences. The terminal sequences of candidate inserts were sequenced according to Maxam & Gilbert (1980) and clone pUC4K BTV-10.7 was identified. This clone retained the entire coding region and only eight bases upstream of the ATG translation initiation codon of the S7 cDNA (Yu et al., 1988). The 5' sequence of this clone is shown in Fig. 1.

The S7 DNA was excised from pUC4K BTV-10.7 with *BamHI* and cloned into the *BamHI* site of the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987). The derived recombinant transfer vector (pAcYM1 BTV-10.7) was characterized by restriction enzyme and sequence analyses and was shown to have the BTV S7 gene insert in the correct orientation for expression directed by the AcNPV polyhedrin promoter.

**Transfection and selection of recombinant viruses.** To obtain recombinant baculoviruses that would express the BTV S7 gene, *S. frugiperda* cells were transfected with mixtures of infectious AcNPV DNA and the purified DNA obtained from plasmid pAcYM1 BTV-10.7. Recombinant viruses were obtained by selecting plaques lacking the polyhedrin-positive phenotype. One of the derived recombinant viruses was designated AcBTV-10.7.

**Preparation of infected cell lysates.** For analysis of polypeptides synthesized by the recombinant baculovirus, monolayers of *S. frugiperda* cells in 35mm tissue culture dishes were infected with virus at a multiplicity of 10 p.f.u./cell and incubated at 28 °C for 3 days.

![Fig. 1](image-url)
and AHSV serotype 4 were a gift from Dr B. J. Erasmus, Veterinary Research Institute, Onderstepoort, South Africa.

To obtain polyclonal antiserum against VP7, S. frugiperda cells were harvested 3 days post-infection with AcBTV-10.7 and the proteins resolved by SDS-PAGE. Proteins were located by precipitation with 0.3 M-KCl and the band containing VP7 was excised and crushed in PBS. The resulting slurry was injected intraperitoneally into a rabbit at days 0, 7, 14 and 21 and serum samples were removed at 2 day intervals from days 22 to 30.

Western blot analysis. After electrophoresis, proteins were transferred onto an Immobilon membrane (Millipore) using a semi-dry electrophoretblotter (Kyhse-Andersen, 1984). Membranes were then soaked in blocking buffer (5% skimmed milk powder plus 0.05% Tween 20 in PBS) at 4 °C overnight. For identification of the VP7 of recombinant virus AcBTV-10.7, anti-BTV-10 rabbit serum was used as described previously (Roy et al., 1990a). For reactivity with other BTV serotypes and with EHDV serotypes, the membrane containing viral proteins was incubated with rabbit anti-VP7 serum diluted 1:5000 in blocking buffer containing an alkaline phosphatase conjugate of goat anti-rabbit IgG (Sigma). Following further washes, bound antibody was detected using 5-bromo-4-chloro-3-indolyl phosphate and colour reagent nitro blue tetrizolium chloride (Bethesda Research Laboratories).

Purification of recombinant VP7. AcBTV-10.7-infected S. frugiperda cells were pelleted by low-speed centrifugation, rinsed in PBS and resuspended at 5 x 10^7 cells/ml in 10 mM-Tris-HCl pH 7.5, containing 0.5% NP40. Nuclei were then removed by centrifugation at 2000 r.p.m. for 10 min.

Ice-cold saturated ammonium sulphate in 100 mM-Tris-HCl pH 7.5 was added to the cytoplasmic extract to a final saturation of 20%. The precipitated protein was pellet centrifugation and resuspended in 10 mM-Tris-HCl pH 7.5. Insoluble material was removed by low-speed centrifugation and the supernatant dialysed overnight against 10 mM-Tris-HCl pH 7.5. This preparation was loaded onto a DEAE-Sephacel column in the same buffer and bound proteins were eluted using a salt gradient of 0 to 750 mM-NaCl in 10 mM-Tris-HCl pH 7.5. Fractions containing VP7 were identified by SDS-PAGE.

ELISA test using purified VP7. Each well of a 96-well, PVC microtitre plate (Flow Laboratories) was coated overnight at 4 °C with 50 μl VP7 (i.e. approximately 100 ng per well) in 0.1 M-sodium bicarbonate, pH 9.6. After absorption of the antigen, the plates were washed four times in blocking buffer (0.05% Tween 20, 0.3% BSA in PBS) and incubated with 50 μl/well of serial dilutions of reference anti-BTV or anti-AHSV-4 guinea-pig sera (1:50 to 1:3200) in blocking buffer for 1 h at room temperature. After four washes in PBS containing 0.05% Tween 20, the plates were incubated with 50 μl/well of a 1:400 dilution of anti-guinea-pig IgG-horseradish peroxidase conjugate for 1 h at room temperature. Following washing as before, the plates were then developed by adding 50 μl of substrate [o-phenylenediamine (OPD); Sigma] in substrate buffer (1 mg OPD per ml in 0.1 M-sodium citrate buffer pH 4.5). The absorbance at 492 nm was measured using a multichannel spectrophotometer.

Results

Construction and isolation of a recombinant baculovirus

A recombinant baculovirus containing the BTV-10 S7 sequence was constructed as described under Methods.

To facilitate the original cloning representing cDNA of RNA S7 into pBR322, homopolymeric tails had been added (Yu et al., 1988). These sequences are possibly detrimental to high level expression (D. H. L. Bishop, personal communication) and so were removed by digestion with the exonuclease Bal 31.

Previous studies have demonstrated that for many genes, high expression with a recombinant baculovirus is obtained using the transfer vector pAcYM1 (Emery & Bishop, 1987; Matsuura et al., 1987; Urakawa & Roy, 1988; Roy et al., 1990a, b; Thomas et al., 1990). This vector contains the entire upstream sequence of the AcNPV polyhedrin gene, including the A of the polyhedrin translation initiation codon. The DNA copy containing the entire coding region of the S7 gene was therefore inserted into the BamHI site of this vector and the recombinant transfer vector pAcBTV-10.7 was identified. Subsequently, recombinant viruses (e.g. AcBTV-10.7) were obtained by transfecting S. frugiperda cells with a mixture of this plasmid DNA and infectious wild-type AcNPV DNA as described in
Methods. The presence of the S7 gene in one of the recombinant viruses (AcBTV-10.7), was confirmed by subjecting the viral DNA to Southern blot analyses as described previously (Inumaru & Roy, 1987; data not shown).

High level expression and characterization of BTV-10 VP7 in S. frugiperda cells

To demonstrate that the VP7 of BTV-10 was synthesized in cells infected with the recombinant baculovirus AcBTV-10.7, a sample of extracted protein was analysed by SDS–PAGE together with samples of mock-infected and wild-type AcNPV-infected cells; the protein bands were detected by staining with Coomassie blue as described in Methods.

As shown in Fig. 2, the major protein synthesized by cells infected with AcBTV-10.7 had an Mr of 38K, i.e. similar to that predicted from the amino acid sequence of the S7 gene product (Yu et al., 1988), and similar to that of the authentic VP7 protein band recovered from BTV. The equivalent protein band was not present either in mock-infected cells nor in cells infected with wild-type AcNPV. The expression level of the VP7 protein was estimated at approximately 100 mg/litre of infected cells (2 × 10⁹ cells).

To confirm that the 38K protein was indeed a BTV-10 gene product, a duplicate gel was subjected to Western blot analyses using polyclonal anti-BTV-10 antisera as described in Methods. The positive signals obtained confirmed that the expressed 38K band was an authentic BTV gene product (see below).

Purification of the recombinant VP7 protein

The expressed VP7 was found to be present in the cytoplasmic extract of S. frugiperda cells infected with AcBTV-10.7 (Fig. 3a, lane 2). Subsequent precipitation with 20% saturated ammonium sulphate gave a protein fraction that contained predominantly VP7 as judged by SDS–PAGE analysis (Fig. 3a, lane 3). Further purification by anion-exchange chromatography using DEAE–Sephacel gave a peak eluting at a NaCl concentration of 200 mM. This material gave a single, major band corresponding to 38K on SDS–PAGE, with very little evidence of contaminating protein (Fig. 3a, lane 4). To verify that the purified protein was VP7, aliquots of this material were subjected to Western blot analyses using anti-BTV-10 antisera. As a positive control, the crude recombinant virus-infected cell lysate was also included. As shown in Fig. 3(b), 38K bands of both crude and purified samples reacted strongly with anti-BTV-10 antisera. Similar positive signals were obtained when polyclonal anti-BTV antibodies (see below) and a monoclonal antibody to VP7 of BTV-10 (data not shown) were used for binding purified VP7 in ELISAs.
**Demonstration of the group-specific antigenicity of the baculovirus-expressed VP7 protein and application of purified VP7 as a diagnostic reagent**

VP7, the major core protein of BTV, has been identified as a group-specific antigen (Huismans & Erasmus, 1981; Gumm & Newman, 1982; Hübschle & Jang, 1983). However, the hybridization studies have indicated that the VP7 gene is the least well conserved by comparisons with three other inner capsid protein genes (VP1, VP3, and VP4; Ritter & Roy, 1988). To determine whether the VP7 gene product is indeed a group-specific antigen, we prepared a rabbit monospecific, polyclonal antiserum to the expressed VP7 and used it in Western blot assays with purified BTV. Since high serological cross-reactivity between BTV and EHDV was observed previously, EHDV serotypes were also included in the assays (Knudson & Monath, 1990). Five of the U.S. BTV serotypes (serotypes 2, 10, 11, 13, and 17), and EHDV-1 and -2, were purified as described by Mertens et al. (1987) and the polypeptides were analysed by SDS-PAGE (Fig. 4a), followed by Western blot analyses as described in Methods. As shown in Fig. 4(b), the monospecific anti-VP7 serum reacted strongly with the VP7 proteins of all five BTV serotypes. In addition, clear signals were obtained with both EHDV-1 and -2 serotypes, indicating that the VP7 of these two virus groups are related to each other.

In order to evaluate VP7 further as a diagnostic reagent, purified VP7 was coated onto microwell plates and tested by ELISA for the presence of anti-VP7 antibodies in polyclonal antisera representing all 24 BTV serotypes (BTV-1 to -24). Serial dilutions of each serum (1:50 to 1:3200) were tested to determine the VP7 antibody titres. Fig. 5 shows the results of the analyses expressed as dilutions of sera giving 50% of the maximum absorbance at 450 nm. As expected, recombinant VP7 was recognized by all the BTV antisera tested in the ELISA.

To determine whether BTV VP7 proteins share any epitopes with that of AHSV, a distantly related orbivirus, a reference antiserum from AHSV-4-infected guinea-pigs was also tested. As shown in Fig. 5 a clear positive signal was obtained with the AHSV serum, albeit approximately 10-fold lower than the signals obtained with BTV antisera at equivalent dilutions. Nevertheless, the signals were significantly higher than those obtained with the control, uninfected guinea-pig sera. Taken together the results indicate that the inner capsid protein, VP7 of BTV, is valuable in the identification of orbivirus infection but perhaps could not be used to discriminate between BTV, EHDV and AHSV.

**Discussion**

The smaller of the two major core proteins of BTV, VP7, has been expressed at high level in *S. frugiperda* cells using a recombinant baculovirus. Verwoerd et al. (1979)
Fig. 5. ELISA with purified VP7 antigen. Purified VP7 was bound to the solid phase and sera from guinea-pigs immunized with different BTV serotypes (BTV-1 to BTV-24) or AHSV-4 were titrated against it as described in Methods. The reactivity of different antisera with VP7 antigen is expressed as dilution of antiserum or normal guinea-pig (GP) serum giving 50% of the maximum absorbance (at 450 nm).

suggested that this capsid protein is the group-specific antigen of BTV. VP7 of serotype 1, purified from culture supernatant fluids of infected BHK cells, was subsequently shown to react with antisera to both BTV types 1 and 4 (Gumm & Newman, 1982). The S7 gene which encodes VP7 is conserved among BTV serotypes (Ritter & Roy, 1988) and we have confirmed that VP7 is indeed a group-specific antigen by both ELISA and Western blot analysis. Antisera raised against BTV particles of various serotypes reacted with expressed VP7 and conversely antiserum raised against expressed VP7 of BTV-10 also reacted with VP7 from BTV of all the serotypes tested. Using established serological methods to characterize orbiviruses, such as complement fixation, immunofluorescence, agar gel precipitation tests and neutralization tests, all involving whole virions as antigens, confusion can arise due to various levels of cross-reaction between serotypes and serogroups (Knudson & Monath, 1990). The use of purified antigens, such as VP7, should help to overcome this confusion and establish which antigens are truly type-specific, as VP2 has been shown to be (Huismans & Erasmus, 1981), and which have wider specificities.

Our results show that VP7 is antigenically very similar in all BTV serotypes as would be expected from the results of cross-hybridization of RNA segments (Ritter & Roy, 1988). In this context it is noteworthy that Northern blot hybridization studies indicated that the sequences of RNA S7 of some BTV serotypes (e.g. BTV-7 and BTV-19) may differ slightly from that of BTV-10. However sequence differences in the genes did not reflect on the cross-reactivity of the gene products. In fact, antisera against each of the 24 serotypes appeared to cross-react equally well with BTV-10 VP7 protein. Similarly, although the sequences of the corresponding RNA segments of EHDV serotypes 1 and 2 bear little homology to the S7 RNA of BTV (Ritter & Roy, 1988), the respective gene products clearly share one or more epitopes, as the antiserum raised to the expressed VP7 reacts with the VP7 proteins of both virus types. Since EHDV is the most closely related orbivirus to BTV, the conserved nature of the core antigen VP7 would be expected. In fact VP7 of both viruses interacts equally well with VP3, the second major core antigen of EHDV-1, resulting in viral core-like particles (P. Roy, unpublished results).

Much to our surprise, antiserum against AHSV also gave a positive signal against recombinant VP7 on ELISA. Although the titre was much lower than that of any of the anti-BTV antisera, it was clearly above the background and suggests some degree of similarity between VP7 of these two groups of viruses. Cross-complement fixation tests or agar gel precipitation tests using crude viral antigens revealed no serological relationships between BTV and AHSV (Erasmus et al., 1970). Similarly, Bremer et al. (1990) were unable to detect any cross-hybridization between DNA clones of AHSV and BTV RNA segments. It will be interesting to determine whether purified VP7 of BTV can react with the antisera of any other orbiviruses.
The S7 gene has been sequenced previously and the derived amino acid sequence is very hydrophobic (Yu et al., 1988). This has been exploited in the purification procedure by using a low percentage ammonium sulphate precipitation as the first step. Subsequent ion-exchange chromatography gave material that was judged to be at least 95% pure.

The hydrophobicity of VP7 may well be important for its interaction with VP3 to form cores. The isolated nucleocapsid protein, one of the major structural proteins of rotavirus, has been shown to form hexagonal lattices, single-shelled structures and tubules (Ready & Sabara, 1987). No such assembly has been seen with the nucleocapsid protein, one of the major structural proteins of BTV cores have a double-shelled structure. Studies of B. V. V. Prasad (personal communication) have shown that BTV cores have a double-shelled structure. Single-shelled structures and tubules (Ready & Roy, 1990). In the absence of VP3, correct VP7 protein-protein interactions may be unable to form and hydrophobic interactions between VP7 molecules may then lead to non-specific aggregation. Huismans et al. (1987b) have isolated subcore particles composed predominantly of VP3 and the cryoelectron microscopic studies of B. V. V. Prasad (personal communication) show that BTV cores have a double-shelled structure. VP7 has been shown to be on the outside of core particles (Hyatt & Eaton, 1988). It is possible that nascent VP7 is laid down as the outer layer onto an existing subcore particle. The ability to purify large amounts of both recombinant VP7 and core-like particles enables structural studies to be performed on the isolated protein and on the protein when bound to VP3, to elucidate further the nature of the interactions and pathway of virus morphogenesis.

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References


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