Expression of Bluetongue Virus Group-specific Antigen VP3 in Insect Cells by a Baculovirus Vector: Its Use for the Detection of Bluetongue Virus Antibodies

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SUMMARY
DNA representing RNA segment 3 of bluetongue virus (BTV) serotype 17, corresponding to the gene that codes for a group-specific antigen VP3, has been inserted into a baculovirus transfer vector in lieu of the 5' coding region of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus (AcNPV). After cotransfection of Spodoptera frugiperda cells with wild-type AcNPV DNA in the presence of the derived recombinant transfer vector DNA, polyhedrin-negative recombinant baculoviruses were recovered. When S. frugiperda cells were infected with one of these recombinant viruses, a protein that was similar in size and antigenic properties to the BTV VP3 protein was synthesized. Antibodies raised in mice or rabbits to the baculovirus-expressed VP3 protein immunoprecipitated the VP3 protein of BTV-17 as well as that of BTV-10. The expressed antigen reacted with antisera representing four U.S.A. BTV serotypes in an indirect ELISA test.

INTRODUCTION
Bluetongue virus (BTV) is the prototype of the Orbivirus genus (Reoviridae family). The virus contains a segmented genome consisting of 10 double-stranded RNA molecules each of which is unique and codes for at least one polypeptide product (Gorman et al., 1983; Sangar & Mertens, 1983). The RNA genome is surrounded by an inner core of five polypeptides (VP1, VP3, VP4, VP6 and VP7), which in turn is surrounded by an outer capsid of two polypeptides (VP2 and VP5) to give a complete virion particle with a diameter of approximately 70 nm (Martin & Zweerink, 1972; Huismans, 1979; Mertens et al., 1984). The outer capsid protein, VP2, elicits neutralizing antibodies while VP3 and VP7 elicit group-reactive antibodies (Huismans & Erasmus, 1981; Huismans et al., 1979).

We have recently published the complete nucleic acid sequence of RNA segment 3 (L3) from BTV serotypes 10 and 17 (BTV-10, BTV-17; Purdy et al., 1984; Ghiasi et al., 1985). Comparison of these sequences showed them to be highly conserved both at the nucleic acid and amino acid level. The primary gene product (VP3) of these genes differed by only nine amino acids. Using a DNA copy of BTV-17 L3, we have demonstrated hybridization of this probe with the equivalent RNA segments of the 19 BTV serotypes isolated from different geographical locations (Roy et al., 1985).

In order to obtain a high yield of the group-specific VP3 antigen, an essentially complete DNA copy of the L3 RNA segment of BTV-17 has been inserted into the genome of Autographa californica nuclear polyhedrosis virus (AcNPV) in lieu of the 5' coding region of the AcNPV polyhedrin gene (Smith et al., 1983; Possee, 1986). Expression of the BTV-17 VP3 polypeptide by the derived recombinant virus in Spodoptera frugiperda cells is described. The expressed protein has been shown to correspond in size to BTV-induced VP3 protein, to react with antisera
corresponding to BTV-10, -11, -13 and -17 (U.S.A. serotypes) and, in mice or rabbits, to induce antibodies that react with BTV-10 or BTV-17 VP3 antigens.

METHODS

Virus and cells. The AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of S. frugiperda cells in media containing 10% foetal bovine serum according to the procedures described by Brown & Faulkner (1977).

BTV segment 3 DNA. The A-T-tailed, double-stranded cDNA of BTV segment 3 has been cloned into the HindIII site of pBR322 and the complete sequence determined (Purdy et al., 1984). The clone, 2772 nucleotides long (excluding the A-T sequences) was employed to synthesize the 901 amino acid VP3 protein using a baculovirus expression system. The BTV DNA has no internal HindIII or SmaI sites (Purdy et al., 1984).

Construction of AcNPV recombinant transfer vectors. Before ligation into the transfer vector, it was necessary to remove the majority of the terminal A–T base pairs from both ends of the L3 DNA. The viral insert was excised from the original pBR322-derived plasmid by digestion with HindIII and purified by agarose gel electrophoresis and electroelution. The viral DNA was then digested briefly with Bal31 exonuclease, the digestion monitored by the induced changes in the HindIII restriction pattern, and the products repaired with Klenow enzyme. In order to ligate the viral DNA into the unique SmaI site of the AcNPV transfer vector designated pAcRP1S, a SmaI linker was ligated into the viral DNA, the products digested with SmaI and then ligated to the dephosphorylated vector. This vector was derived from the pAcRP1 plasmid, described originally by Possee (1986), by modifying its unique BamHI site with a BamHI–SmaI adaptor. Several clones were obtained with the L3 DNA insertion. Whilst these experiments were in progress it was demonstrated that recombinant viruses derived from the pAcRP1 vector were not as efficient in the expression of foreign genes as those derived from the vector pAcRP6 (Matsuura et al., 1986; Possee, 1986). The L3 viral DNA was therefore excised from pAcRP1S transfer vector (pAcRP1.17.3) by digestion with SmaI and religated into the repaired, dephosphorylated, BamHI site of the modified transfer vector pAcRP6. Recombinant transfer vectors were recovered and characterized by restriction enzyme mapping. The sequence of the coding strand at the insertion site was verified (Maxam & Gilbert, 1980). Recombinant pAcS17.3, was shown to have the BTV DNA in the right orientation for expression by the AcNPV polyhedrin promoter (Fig. 1, 2).

Transfection and selection of recombinant viruses. S. frugiperda cells were transfected with mixtures of purified, infectious AcNPV DNA and pAcS17.3 plasmid DNA essentially as described by Matsuura et al. (1986). After 4 days incubation at 28 °C, the supernatant fluids were harvested and titrated in confluent monolayers of S. frugiperda cells. Plaques exhibiting no evidence of occlusion bodies (viral polyhedra), as determined by transmission light microscopy, were recovered and retitrated on S. frugiperda cells to obtain recombinant, polyhedrin-negative viruses. Following a third plaque picking, high titre stocks (10⁷ to 10⁸ p.f.u./ml) of the recombinant virus (RP6S3.11) were obtained using monolayer cultures of S. frugiperda cells.

Southern blot hybridization. To obtain recombinant virus DNA, S. frugiperda cells were infected with virus at a multiplicity of 10 p.f.u./cell and incubated at 28 °C for 48 h. The infected cells were harvested, briefly sonicated to obtain cell lysates and centrifuged for 10 min at 500 g to remove cell debris. The supernatant fluids were recovered. The procedures used for virus isolation and subsequent viral DNA extraction were essentially similar to those described by Matsuura et al. (1986). DNA was resuspended in water and stored at −20 °C. For Southern analyses, preparations of viral DNA were digested to completion with EcoRV and HindIII and the products resolved by electrophoresis in 0.8 % agarose (Bethesda Research Laboratories) then blotted to 'Genescreen Plus' (New England Nuclear). After drying, DNA was hybridized to nick-translated BTV-17 segment 3 DNA obtained from clone pAcS17.3 (Southern, 1975). The membranes were washed and autoradiographed.

Northern blot hybridization. To obtain infected cell RNA, S. frugiperda cells were inoculated with RP6S3.11 recombinant or with wild-type AcNPV at a multiplicity of 10 p.f.u./cell and incubated for 36 h at 28 °C in medium; RNA extraction, mRNA purification, gel electrophoresis, and Northern blotting procedures were carried out as described by Matsuura et al. (1986). The blotted membranes were then dried, and hybridized to 32P-labelled nick translation products of the appropriate viral DNA as described by Denhardt (1966). After hybridization the membranes were washed and autoradiographed.

Protein and immunoprecipitation analyses. Monolayers of S. frugiperda cells were infected with recombinant virus (RP6S3.11) at a multiplicity of 10 p.f.u./cell in 35 mm tissue culture dishes containing 1.5 x 10⁶ cells and incubated at 28 °C for 48 h. Each dish was then labelled with 100 μCi of [35S]methionine (New England Nuclear, 1154 Ci/mmol) for 2 h in methionine-free medium. BHK-21 cells were similarly infected with BTV-10 at a multiplicity of 10 p.f.u./cell and, after 18 h of incubation at 37 °C, the cells were labelled with 75 μCi of [35S]methionine for 2 h in methionine-free medium. Prior to labelling, the cells were incubated for 1 h in methionine-free medium to reduce the intracellular pools of the precursor. After the labelling periods, the medium was removed, the monolayers were rinsed three times with cold phosphate buffered saline (PBS) and the cells lysed in 500 μl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M-Tris–HCl, 0.01 M-
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EDTA, 0.1% SDS, pH 7.4). Samples of 10 μl of the extracts were incubated with 5 to 10 μl of the appropriate antiserum for 1 h at room temperature before the addition of 25 μl of a suspension of 100 mg of Protein A-Sepharose CL-4B beads (Sigma) in 1 ml RIPA buffer. Following a further 1 h incubation at room temperature, the beads were recovered by centrifugation, washed three times with cold RIPA buffer and the immune complexes that were bound to the beads were recovered by boiling for 5 min in dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM-Tris-HCl, 0.01% bromophenol blue, pH 6.8) followed by centrifugation. Samples of the supernatant fluids were subjected to electrophoresis in 10% discontinuous gels of polyacrylamide as described by Laemmli (1970). After electrophoresis, the gels were impregnated with fixing solution (40% methanol, 10% acetic acid in water), dried and exposed at −70 °C to X-ray film.

Production of antibodies in mice and rabbits. S. frugiperda cells infected with the RP6S3.11 recombinant were collected and subjected to freezing and thawing, followed by low speed centrifugation. The supernatant fluids were used to raise antibodies in mice or rabbits. For mice, each animal received one intraperitoneal injection of 50 μg antigen in Freund's complete adjuvant on day 0, followed by three injections of 50 μg antigen in Freund's incomplete adjuvant on days 7, 14 and 21 and one intraperitoneal injection of 2 × 10^6 Ehrlich's ascites cells on day 18. Ascitic fluids were removed at intervals from day 22 to day 34. For rabbits, each animal received one intramuscular injection of 500 μg antigen in Freund's incomplete adjuvant on days 7 and 16. Blood was collected on day 14 and on day 22.

Immunoprecipitation of proteins using mouse ascitic fluids or rabbit antisera. The mouse ascitic fluids and rabbit sera were diluted 25-fold with RIPA buffer and incubated with [35S]methionine-labelled, BTV-10-infected BHK-21 cell lysates for 1 h prior to immunoprecipitation and gel analyses as described above.

Indirect ELISA. A solid-phase, indirect micro-ELISA using rigid, flat-bottomed polystyrene ELISA plates (Nunc, Immunoplate I) was used for examination of recombinant virus RP6S3.11 antigen reactivity with polyclonal BTV antisera. S. frugiperda cells infected with the RP6S3.11 recombinant were collected and subjected to freezing and thawing followed by low speed centrifugation. The supernatant was diluted (1:10 to 1:10000) with carbonate buffer (15 mM-Na2CO3, 35 mM-NaHCO3, pH 9.6) and 100 μl of each diluted preparation was added to each well of an ELISA plate and left overnight at 4 °C. The plates were washed three times between each step by flooding the wells of the plate with PBST buffer (0.0025% of Tween 20 in PBS). The BTV antisera were diluted (1:1000) in PBST containing egg albumin (0.2% w/v final concentration) and polyvinylpyrrolidone, mol. wt. 44000 (2% w/v final concentration). The diluted antisera (100 μl in each well) were added to the adsorbed antigen and incubated at 37 °C for 1 h. After washing of the plate, 100 μl of 1:100 dilution of alkaline phosphatase-conjugated Protein A (Sigma) was added to each well followed by 2 h incubation at 28 °C. The substrate, p-nitrophenyl phosphate (Sigma) solution (0.1% w/v final concentration) was added, for 30 min or less at room temperature. After suitable colour development the reaction was stopped by the addition of 75 μl of 3 M-NaOH. The absorbance was read using a multichannel spectrophotometer (Titertek Multiskan, Flow Laboratories) at a wavelength of 405 nm.

RESULTS

Construction of the recombinant virus (RP6S3.11)

The strategy for the construction of the baculovirus transfer vector containing the entire VP3 gene is shown in Fig. 1. The procedure involved the removal of the majority of the terminal dA–dT sequences and insertion of the DNA into a baculovirus transfer vector as described in Methods. The orientation and sequence of the L3 gene in the transfer vector relative to the AcNPV polyhedrin leader were determined by DNA sequence analysis (Fig. 2) as described in Methods. For the recombinant transfer vector pAcSI17.3 the VP3 DNA insert contained one T nucleotide (representing the terminal residue of the dA–dT tails) followed by the entire L3 sequence including the terminal non-coding regions (Purdy et al., 1984). In order to transfer the L3 gene into the AcNPV genome, S. frugiperda cells were cotransfected with pAcSI17.3 DNA and then recombinant viruses were isolated from the infected cells by selecting progeny with a polyhedrin-negative plaque phenotype (0.1 to 1% of the polyhedrin-positive plaques) followed by three successive cycles of plaque purification. By this means stocks of recombinant virus (RP6S3.11) were obtained.

Analysis of the recombinant viral DNA by restriction enzyme cleavage and Southern blot hybridization

Recombinant virus RP6S3.11 was propagated, purified, and the DNA was extracted as described in Methods. The viral DNA was cleaved with the restriction endonucleases EcoRV and HindIII since the vector contains an EcoRV site at the residue 85 upstream of the 5' insertion
Fig. 1. Construction of the pAcSI17.3 recombinant baculovirus transfer vector containing the BTV-17 VP3 gene. Details of the preparation of the recombinant transfer vector are given in Methods.

site and a HindIII site downstream of the 3' insertion site. The fragments were then separated by electrophoresis in 0.8% agarose gel in order to identify the L3 DNA. The DNA fragments were transferred to 'Genescreen Plus' membrane (Southern, 1975) and the L3 gene was identified by hybridization to nick-translated L3 DNA excised from pAcSI17.3. As shown in Fig. 3, the RP6S3.11 recombinant virus DNA contained sequences which hybridized with the L3 gene. Since segment 3 DNA has one internal EcoRV site at the nucleotide residue 1736 (Purdy et al., 1984), two hybridized bands (approximately 1.8 kb and 1.2 kb in size) were visualized in both the RP6S3.11 and pAcSI17.3 lanes. As expected, AcNPV DNA did not hybridize to the probe (Fig. 3).
Expression of BTV-17 VP3 in S. frugiperda cells

Infection of S. frugiperda cells with the recombinant viruses did not apparently produce nuclear or cytoplasmic inclusions (data not shown). The presence of mRNA species representing the BTV gene in cells infected with the RP6S3.11 recombinant virus was determined by Northern blot analyses, as described in Methods. As shown in Fig. 4,
polyadenylated RNA recovered from cells infected with the recombinant virus hybridized to the 
$^{32}$P-labelled nick-translated L3 DNA probe. This BTV-specific mRNA was estimated to be approxi-
ately 3.8 kb in size (i.e. corresponding to the 2.8 kb BTV gene plus 1 kb of the AcNPV poly-
hedrin gene sequence; Possee, 1986). An RNA band of 1200 bp was identified in the AcNPV-infected cells when a 545 bp probe representing the AcNPV polyhedrin gene was employed (Howard et al., 1986). It was concluded, therefore, that BTV-related mRNA species were synthesized in the recombinant virus-infected cells.

In order to determine whether BTV-17 VP3 was synthesized in the recombinant virus-
infected cells, S. frugiperda cells were infected at high multiplicity with AcNPV, or the recombinant baculovirus RP6S3.11 and pulse-labelled with $[^{35}S]$methionine as described in Methods. A sample of the recombinant virus lysate was immunoprecipitated with polyclonal BTV-17 or BTV-10 antiserum prior to resolution by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 5, both uninfected and recombinant virus-infected cell lysates lacked the 33000 mol. wt. polyhedrin protein induced by AcNPV (Hooft van Iddenkinge et al., 1983; Vlak et al., 1981). Analyses of the immunoprecipitated protein included as controls BHK cells infected with either BTV-10 or BTV-17 as well as immunoprecipitates of S. frugiperda cell lysates infected with a baculovirus recombinant, RP6S2.2, containing the BTV-10 VP2 protein (mol. wt. approx. 93000; Inumaru & Roy, 1987). As shown in Fig. 5, both BTV-17 and BTV-10 antisera precipitated a protein with mol. wt. approximately 92500 from the recombinant virus-infected cell lysates. These results indicated that BTV-17 VP3 protein was expressed during the recombinant virus infection and reacted with either anti-BTV-17 or anti-BTV-10 sera.

Immunoprecipitation of BTV-induced VP3 by ascitic fluids derived from mice immunized with the baculovirus-expressed VP3 protein

To assess the ability of the VP3 produced by the recombinant baculovirus to induce antibodies which could react with VP3 antigen present in BTV-infected BHK-21 cells, mice were immunized with recombinant RP6S3.11 virus-infected S. frugiperda cell extracts as described in Methods. Ascitic fluids were collected between 22 and 34 days post-immunization and were used in immunoprecipitation tests to determine whether they contained antibodies that would react with the VP3 antigen present in BTV-10- or BTV-17-infected BHK-21 cells. Immunoprecipitation studies of viral polypeptides using BTV-10- (data not shown) or BTV-17-
infected BHK-21 cell extracts were performed with either immunized (Fig. 6, anti-RP6S3.11), or non-immunized mouse ascitic fluids (Fig. 6, control) and the precipitated proteins were compared to the proteins precipitated with polyclonal BTV-17 antisera (Fig. 6, anti-BTV-17). The immunized mouse ascitic fluids, but not the control material, precipitated VP3 from BTV-
17-infected BHK-21 cell extracts.

Reactivity of the baculovirus-expressed VP3 antigen with BTV antisera using an ELISA test

In order to determine whether VP3 protein produced by the recombinant baculovirus could react with homologous or heterologous BTV antisera, the recombinant virus-infected S. frugiperda cell extracts were adsorbed to microtitre plates as described in Methods. The adsorbed antigen was then incubated with either polyclonal BTV-17 or BTV-10 antiserum or with non-immune serum as described in Methods. As a control, AcNPV-infected cell extracts were also employed and incubated with each of the sera. The derived antigen–antibody complexes were then detected by incubating with alkaline phosphatase–Protein A conjugate followed by the addition of a substrate as described in Methods. As shown in Fig. 7, both BTV antisera (1:1000 dilution) reacted with recombinant antigen to a high level in contrast to the reaction with AcNPV antigen. Normal rabbit serum did not react to any significant level either with the recombinant antigen or with wild-type AcNPV antigen.

DISCUSSION

Recombinant baculoviruses containing the segment 3 gene of BTV-17 have been constructed and used to express the group-specific antigen of BTV in cells of S. frugiperda.
Fig. 5. Expression of BTV-17 VP3 protein by a recombinant baculovirus. Uninfected *S. frugiperda* cells (lane 1) or cells infected with wild-type AcNPV (lane 2) or with a baculovirus recombinant RP6S2.2 containing the BTV-10 VP2 protein (lane 3) or with a recombinant virus RP6S3.11 (lane 4), were pulse-labelled after 48 h with [35S]methionine, as described in Methods. Lysates of the infected or uninfected cells were electrophoresed in a 10% polyacrylamide gel and the labelled polypeptides were identified by fluorography. The positions of the AcNPV polyhedrin protein, BTV-10 VP2 and BTV-17 VP3 proteins are indicated (see text). An aliquot of RP6S2.2 recombinant lysate was immunoprecipitated with polyclonal BTV-10 (lane 5) and similarly an aliquot of RP6S3.11 was immunoprecipitated with polyclonal BTV-17 (lane 9) or BTV-10 (lane 10) and each sample was resolved by 10% polyacrylamide gel electrophoresis along with the immunoprecipitated samples of BTV-10 (lane 7) and BTV-17 (lane 8) infected BHK cells with homologous antisera.

Immunoprecipitation of the VP3 protein produced in the insect cells showed that the product was similar to the BTV VP3 both in antigenicity and in size. The expressed VP3 antigen was also shown to elicit antibodies in mice and rabbits that recognized BTV protein. Furthermore, the
Fig. 6. Detection of BTV-17 proteins in BTV-17-infected BHK-21 cells by antibody raised in mice to extracts of RP683.11 recombinant baculovirus-infected S. frugiperda cells. BHK-21 cells were radiolabelled for 2 h with [35S]methionine at 24 h post-infection with BTV-17 and extracts immunoprecipitated with control (lane 3) or immunized mouse ascitic fluids (lane 2, anti-RP683.11). A sample of BTV-17-infected cell lysate was similarly immunoprecipitated with polyclonal BTV-17 antisera (lane 1).

Fig. 7. Titration of expressed VP3 protein–anti-BTV sera reaction by indirect ELISA. Different dilutions of recombinant virus-infected cell extracts were adsorbed to the solid phase and examined using 1:1000 dilutions of rabbit anti-BTV-10 serum (○—○) or anti-BTV-17 serum (○—○), or with normal rabbit serum (△—△). As controls, AcNPV-infected cell extracts were similarly reacted with anti-BTV-10 serum (●—●), or with anti-BTV-17 serum (○—○) or with normal rabbit serum (△—△).

recombinant virus extract was demonstrated to be reactive with BTV antisera in an indirect ELISA test. Since VP3 is a group-specific antigen, this genetically engineered BTV antigen is being developed as a reagent for the diagnosis of BTV.

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REFERENCES


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