Bluetongue Virus Tubules Made in Insect Cells by Recombinant Baculoviruses: Expression of the NS1 Gene of Bluetongue Virus Serotype 10

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Bluetongue virus (BTV) forms tubules in mammalian cells. These tubules appear to be composed of only one type of protein, NS1, a major nonstructural protein of the virus. To obtain direct evidence for the origin of the tubules, the complete M6 gene of BTV serotype 10 was inserted into the baculovirus transfer vector pAcYM1, so that it was under the control of the polyhedrin promoter of Autographa californica nuclear polyhedrosis virus. After cotransfection of Spodoptera frugiperda cells with wild-type A. californica nuclear polyhedrosis virus DNA in the presence of recombinant transfer vector DNA, polyhedrin-negative baculoviruses were recovered. When S. frugiperda cells were infected with one of the derived recombinant viruses, a protein similar in size and antigenic properties to the authentic BTV NS1 protein was made (representing ca. 50% of the stained cellular proteins). The protein reacted with BTV antibody and formed numerous tubular structures in the cytoplasm of S. frugiperda cells. The tubular structures have been purified to homogeneity from infected-cell extracts by gradient centrifugation. By enzyme-linked immunosorbent assay, the recombinant virus antigen has been used to identify antibodies to five United States BTV serotypes in infected sheep sera, indicating the potentiality of the expressed protein as a group-reactive antigen in the diagnosis of BTV infections.

The genus Orbivirus within the family Reoviridae consists of viruses with similar morphological and physicochemical properties. Bluettongue virus (BTV) is the prototype virus of the Orbivirus genus. It is vectored to vertebrates by Culicoides species. The viruses cause disease in certain ruminants. The virosis of orbiviruses are more fragile than those of reoviruses (20, 21). Unlike reoviruses, orbiviruses have an outer protein shell composed of viral proteins no. 2 and 5 (VP2, VP5) with no well-defined capsomeric structure (20, 21). The complex inner shell of 32 ring-shaped protein capsomers is arranged in icosahedral symmetry and surrounds the 10 double-stranded RNA species (1, 14). This inner core structure contains five types of proteins, two that are major (VP3 and VP7) and three that are minor components (VP1, VP4, and VP6). In addition, in BTV-infected cells three nonstructural proteins are made (NS1, NS2, and NS3) (6). Their function in the replication or morphogenesis of BTV is not known. Two virus-specific entities, tubules and granular inclusion bodies, are routinely observed in BTV-infected cells (13). These morphological structures are attached to the intermediate filament component of the cell's cytoskeleton (3) and are presumed to be involved in the virus assembly process. Huismans and Els (8) have shown that the tubular structures are composed entirely of one type of polypeptide (the 64-kilodalton [kDa] NS1 protein) that is the gene product of BTV middle-size segment no. 6 (M6).

To obtain direct proof that NS1 protein forms tubules, we have expressed the BTV serotype 10 (BTV-10) M6 gene product in an insect baculovirus expression vector derived from Autographa californica nuclear polyhedrosis virus (AcNPV). The BTV-10 NS1 protein expressed by recombinant baculoviruses reacts with BTV antibody and induces numerous tubular structures in the cytoplasm of Spodoptera frugiperda cells.

MATERIALS AND METHODS

Viruses and cells. United States prototype BTV-10 was plaque cloned using monolayers of BHK-21 cells. AcNPV and recombinant baculovirus stocks were grown and assayed in confluent monolayers of S. frugiperda cells in medium containing 10% fetal calf serum, according to the procedures described by Brown and Faulkner (2). Occasionally virus stocks were made using spinner cultures of these insect cells.

DNA manipulations and construction of a complete BTV M6 gene. Plasmid DNA manipulations were carried out following the procedures described by Maniatis and associates (16). Restriction enzymes, T4 DNA ligase, and the Klenow large fragment of DNA polymerase were purchased from New England Biolabs, Inc. (Beverly, Mass.). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim (Federal Republic of Germany).

The construction of plasmid pBTV10-6, representing the complete M6 DNA, is illustrated in Fig. 1. Two M6 DNA clones, representing nucleotides 1 to 1100 (no. 14) and 700 to 1769 (no. 39), were used for this construction (15). Clone 14 was initially recloned in the opposite orientation into the PstI site of pBR322. The derived clone (no. 14-9) was then digested with PvuI, and a fragment of DNA containing M6 residues 1 to 898 was recovered and dephosphorylated (Fig. 1). A PvuI-derived fragment was obtained from clone 39, containing M6 residues 899 to 1769. The two fragments were ligated together. After transformation of Escherichia coli MC1061 cells and the selection of drug-resistant colonies,

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FIG. 1. Schematic diagram of the construction of the transfer vector pAcBTV10-6. A pBR322-based plasmid (pBTV10-6) containing the entire coding region of the BTV-10 M6 DNA was constructed from two overlapping partial clones as described in Materials and Methods. The complete clone (pBTV10-6) was used to construct the transfer vector (pAcBTV10-6) as described in Materials and Methods. The sequence of the 5' insertion site was determined by the method of Maxam and Gilbert (18), using a HindIII restriction fragment (BTV DNA residue no. 296).
followed by colony hybridization screening (5) using the nick-translated products of M6 DNA derived from clones 14 and 39, clone pBT10-6 was identified and confirmed by the appropriate restriction enzyme and sequence analyses (15) to contain BTV-10 M6 residues 1 to 1769.

**Construction of a recombinant baculovirus transfer vector.** A 1,746-base-pair DNA fragment, containing the entire coding region of the BTV-10 M6 DNA, was excised from plasmid pBT10-6 with *Mae*I, repaired with the Klenow large fragment of DNA polymerase, and then cloned into the *Bam*HI site of the baculovirus transfer vector pAcYM1 (17). The derived recombinant transfer vector (designated pAcBT10-6; Fig. 1) was characterized by restriction enzyme and sequence analyses (18) and was shown to have the viral insert in the correct orientation for expression directed by the AcNPV polyhedrin promoter.

**Transfection and selection of recombinant viruses.** To obtain recombinant viruses that would express the BTV M6 gene, *S. frugiperda* cells were transfected with mixtures of infectious AcNPV DNA and DNA obtained from plasmid pAcBT10-6. Recombinant viruses were obtained as described previously (9). One of the derived recombinant viruses was designated AcBT10-6.

**Analysis of infected-cell polypeptides by SDS-PAGE.** *S. frugiperda* cells in 35-mm tissue culture dishes were infected with viruses at a multiplicity of 10 PFU/cell, and the cells were incubated at 28°C for 3 days. At the end of the incubation period, cells were rinsed three times with phosphate-buffered saline (PBS) and suspended in 100 μl of 10 mM Tris hydrochloride buffer (pH 7.4). A 50-μl volume of protein dissociation buffer (2.3% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris hydrochloride, and 0.01% bromophenol blue; pH 6.8) were added to each sample, and the mixture was heated at 100°C for 10 min. Proteins were analyzed by electrophoresis in a 10 to 30% linear gradient polyacrylamide gel (PAGE) in the presence of SDS as described by Laemmli (12). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue.

**Immunoblotting analyses.** After SDS-PAGE, cellular proteins were transferred electrophoretically for 3 h at 0.8 mA/cm² (10) into a Durapore membrane (Millipore Corp.), using a semi-dry electrobolter (Sartoblot II, Sartorius, Corp.). After transfer, the membrane was soaked overnight at 4°C in blocking buffer (5% skim milk and 0.05% Tween-20 in PBS, pH 7.4). The membrane was then treated for 2 h with rabbit anti-BTV-10 serum diluted 1,000-fold in blocking buffer. After a second wash with 0.05% Tween-20 in PBS, the membrane was soaked for 1 h at room temperature in anti-rabbit immunoglobulin G-goat immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Co.), also diluted in blocking buffer. After further washing with PBS-0.05% Tween-20, bound antibodies were detected by incubation using Fast BB salt and β-naphthyl phosphate (Sigma Chemical Co.) as a substrate.

**Electron micrographs of infected cells.** *S. frugiperda* cells were infected with recombinant baculoviruses at a multiplicity of 10 PFU/cell and incubated at 28°C for 3 days. The cells were washed with PBS, fixed with glutaraldehyde, and treated with osmic acid. Cell sections were observed in a JEOL electron microscope.

**ELISA for the detection of antibody to BTV NS1 polypeptide, using recombinant baculovirus-derived antigens.** A solid-phase indirect micro-enzyme-linked immunosorbent assay (ELISA) was used to demonstrate the reactivity of recombinant AcBTV10-6 antigen with various polyclonal BTV antisera (obtained from sheep inoculated with partially purified viruses). *S. frugiperda* cells infected 72 h previously with recombinant AcBTV10-6 virus (see above) were collected and subjected to freezing and thawing, followed by low-speed centrifugation to remove cellular debris. The supernatant, containing solubilized NS1 protein, was diluted (1:10 to 1:10,000) with sodium carbonate buffer (15 mM Na₂CO₃, 36 mM NaHCO₃, pH 9.6). A 96-well polystyrene microplate (Flow Laboratories) was coated overnight at 4°C with 50 μl of the diluted antigen. The plate was washed three times after each step of the following protocol by flooding the wells of the plate with PBS-0.05% Tween-20 buffer. The antigen-coated microplate was washed and blocked with blocking solution for 3 h at room temperature. BTV antisera were diluted (1:100 to 1:12,800) in blocking solution, and 50 μl of diluted serum was added to each well. After 2 h of incubation, the plate was washed and 100 μl of a 1:1,000 dilution of anti-sheep immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemicals Co.) was added to each well, followed by 2 h of incubation at 28°C. The substrate, p-nitrophenylphosphate (Sigma Chemicals Co.) solution (0.1% [wt/vol] final concentration), was added for 30 min or less. After suitable color development at room temperature, the reaction was stopped by the addition of 0.3 M NaOH. The optical density was read using a multichannel spectrophotometer (Microelsa Autoreader, MR 580, Dynatech Co.) at a wavelength of 405 nm.

**Purification of tubular structures by gradient centrifugation.** Cells were infected with the AcBTV10-6 recombinant baculovirus, and the infected cells were harvested 4 or 5 days postinfection. The cells were recovered, washed twice with PBS, suspended in 10 mM Tris hydrochloride (pH 7.4),
RESULTS

Construction of baculovirus recombinants. A plasmid (pBTV10-6) containing the complete BTV-10 M6 sequence was constructed from two overlapping clones as described in Materials and Methods (Fig. 1). To remove unnecessary sequences (such as the homopolymeric tails), the restriction enzyme MaeI was used to isolate the entire coding region of the M6 DNA (including 19 bases upstream from the ATG initiation codon and 59 bases downstream from the TAG stop codon), and the DNA was used to prepare a recombinant baculovirus transfer vector (15).

Previous studies have demonstrated that for many genes the highest baculovirus expression levels are obtained with the transfer vector pAcYM1 (4, 17, 19). This vector contains the entire upstream sequence of the AcNPV polyhedrin gene, including the A of the initiating ATG codon. The coding sequences of the BTV-10 M6 DNA were therefore inserted into the transfer vector pAcYM1 as described in Materials and Methods (Fig. 2). The derived recombinant (pAcBTV10-6) was analyzed by restriction endonuclease digestion, and the junction sequences were determined (Fig. 1). S. frugiperda cells were transfected with mixtures of AcNPV DNA and the plasmid DNA pAcBTV10-6. Recombinant viruses (e.g., AcBTV10-6) were identified by their polyhedrin-negative plaque phenotypes and were plaque purified three times using monolayers of S. frugiperda cells.
Expression of BTV NS1 protein. To demonstrate that BTV-10 NS1 protein was synthesized in recombinant baculovirus-infected cells, protein extracts were prepared from insect cells infected with the recombinant virus AcBTV10-6 as described in Materials and Methods. Extracts were also made from AcNPV- and mock-infected cells. A sample of each preparation was subjected to SDS-PAGE and the resolved proteins were stained with Coomassie brilliant blue. As shown in Fig. 2 (lanes c), the recombinant virus synthesized a protein with a molecular size of ca. 60 kDa, in agreement with the estimated size of the BTV-10 NS1 protein (6). On the basis of comparisons of stained gels containing known quantities of bovine serum albumin, the amount of this protein was estimated to be of the order of 1 mg per 2 \times 10^6 infected cells (unpublished observations), i.e., as high as the polyhedrin protein made in AcNPV-infected cells (compare Fig. 2A, lanes b and c).

To confirm that the 60-kDa protein was NS1, a sample of the AcBTV10-6-infected cell extract was electrophoresed, transferred onto a Durapore membrane, and subjected to Western analysis using anti-BTV-10 sera as described in Materials and Methods. The 60-kDa protein (Fig. 2B, lane c) was identified by the alkaline phosphatase conjugate detection procedure.

Electron microscope analysis of cells infected with recombinants that express the BTV NS1 protein. It has been postulated that the NS1 protein of BTV is the sole component of the tubular structures identified in BTV-infected cells (8). An examination was therefore undertaken of electron micrographs of S. frugiperda cells infected with the AcBTV10-6 recombinant virus or with wild-type AcNPV. In contrast to AcNPV-infected cells (Fig. 3a), numerous tubular structures were evident only in the cytoplasm in the recombinant virus-infected cells (marked T in Fig. 3b through f). In the thin sections, tubules of various lengths were evident. In cross-section, they exhibited a diameter of ca. 60 nm. Many of the tubules appeared to contain ribosomelike particles (Fig. 3, R arrowheads). Both the structures and arrangement of the tubules were comparable to those of the tubular structures reported by others in BTV-infected BHK-21 cells (8). As expected, polyhedra were only evident in the AcNPV-infected cells (Fig. 3a).
Fibrous structures were observed both in AcNPV- and in recombinant virus-infected cells (Fig. 3). In the latter, the tubules appeared to be aligned along the edges of bundles of the fibrous material (see Fig. 3c and d). Both the tubules and the fibrous structures were randomly oriented with respect to one another. In some cells bundles of fibers were seen in both the nucleus and the cytoplasm (Fig. 3e and f), with tubules mostly, but not exclusively, associated with the cytoplasmic fibers. Fewer tubules were seen in the nuclei (Fig. 3f). Whether these tubules were formed in the nucleus, or occurred there because the cell was in the terminal stage of infection, is not known.

To assess the possible three-dimensional arrangement of the tubules in the infected S. frugiperda cells, single arrays in thin sections were examined in a Jeol electron microscope equipped with a tilting stage. A series of photographs were taken at 10° intervals of specimen tilt. It was apparent (Fig. 4 right) that the circular particles visualized in the lower left of the picture taken at a −40° tilt transformed to oblique particles at a −30° tilt and were progressively observed as longitudinal tubules at −10° to +50° tilting stages. Similar transformations were observed for other arrays of tubules in

FIG. 3. Continued.

FIG. 4. Three-dimensional arrangement of the tubules in the infected S. frugiperda cells. (Right) A series of representative virus (panels A through H) of the single arrays in a thin section of the tubules were photographed at 10° intervals of specimen tilt (magnification, × 50,000). (Above) Schematic three-dimensional interpretation of the structures (a, b, and c).
ELISA using recombinant virus-derived NS1 to identify BTV antibodies. Since rabbit antisera against purified BTV had been shown to contain tubule-specific antibodies (8) and since the NS1 gene (M6) of BTV-10 has been shown to be highly conserved among 20 BTV serotypes (7, 19), the question of whether the NS1 protein produced by the recombinant baculovirus reacted with homologous and heterologous BTV antisera was investigated using an ELISA test. Recombinant virus-infected cell extracts were absorbed to microtiter plates (representing some 50 ng of NS1 protein per well) and incubated with either polyclonal BTV-2 or BTV-10, BTV-11, BTV-13, or BTV-17 sheep antisera, or with normal sheep serum as a control. The derived antigen-antibody complexes were detected by incubating with anti-sheep-alkaline phosphatase conjugates followed by the addition of an enzyme substrate. All five BTV antisera reacted with the recombinant antigen in proportion to the endpoint titer of the antiserum (Fig. 5). No reaction was detected with normal sheep serum. No reactivity was obtained when each of the BTV antisera was tested with AcNPV-infected cell extracts or mock-infected cell extracts (data not shown).

Purified tubules recovered from recombinant virus-infected insect cells. To purify the tubular structures, infected-cell extracts were prepared and subjected to sucrose gradient centrifugation. A portion of each fraction was analyzed by SDS-PAGE, which had indicated that there was a heterogenous peak of NS1 protein in the region of 400S. A similar heterogeneous 400S complex of NS1 protein had been identified in BTV-infected mammalian cells (8). The peak fractions containing NS1 protein were pooled and pelleted. The recovered protein, stained with Coomassie brilliant blue, was effectively purified by this one-step procedure (Fig. 6, lane 2). To confirm that the band is indeed BTV NS1, a sample of purified NS1 was subjected to Western analysis using anti-BTV-10 sera as described in Materials and Methods. The protein band is indeed BTV-10 NS1 (Fig. 6, lane 3).

Electron micrographs revealed that the preparation was composed of tubules with little evidence of cytoplasmic or other contamination (Fig. 7).

FIG. 6. SDS-PAGE of purified NS1 protein stained with Coomassie brilliant blue. AcBTV10-6 recombinant baculovirus-infected cells were disrupted, and tubules were recovered by sedimenting by sucrose gradient (10 to 50% [wt/vol] centrifugation as described in Materials and Methods. The peak fractions containing NS1 were pooled and subjected to SDS-PAGE analysis. The proteins recovered from unpurified recombinant virus-infected cells (lane 1) are compared with NS1 protein purified through sucrose gradient centrifugation (lane 2). Lane 3 is the Western blot analysis of purified NS1 protein.

DISCUSSION

Since the NS1 is the major viral protein synthesized in BTV-infected cells and since it is a major constituent of
tubules (8), an investigation was initiated into the ability of baculovirus-expressed NS1 protein to form tubules in insect cells. Two overlapping clones (14 and 39) representing segment M6 (NS1 gene) of BTV-10 were used to construct a plasmid containing the complete gene (pBTV10-6). A recombinant baculovirus was prepared with the NS1 gene sequences under the control of the AcNPV polyhedrin promoter. Data have been presented which show that the BTV NS1 protein is expressed to a high level in insect cells infected with this recombinant baculovirus. From stained preparations of cell extracts it was estimated that the amount of NS1 protein present in cells infected at high multiplicity with the recombinant virus was ca. 50% of the total stainable protein in the cell extract prepared at the end of the infection course. It was demonstrated that the recombinant baculovirus made tubular structures in the insect cells similar to those reported in BTV-infected cell cultures (8). The tubules were purified and demonstrated to be composed of NS1 protein. To what extent the hydrophobic domains and other sequences of the NS1 protein contribute to the structure of the protein and the ability to form tubules can now be investigated by mutational analyses.

The presence of fibrous structures closely associated with the NS1 tubules may be fortuitous. Bundles of fibers are frequently seen in the terminal stages of AcNPV infections. Whether all the fibrous material seen in the recombinant baculovirus-infected cells was AcNPV gene products or was derived from NS1 is not known. Eaton and associates (3) recently reported that a significant proportion of BTV particles were observed to be associated with fibrillar materials containing NS1 when thin sections of cytoskeletons from BTV-infected cells were analyzed. On the basis of that finding it was speculated that perhaps NS1-rich fibrillar material condensed to form tubules and/or that tubules represented a repository of NS1 used in a prior stage of virus morphogenesis. Further studies are needed to determine whether the fibrous structures seen in the recombinant baculovirus-infected cells include NS1 protein and contribute to the formation of tubules.

Previously it has been reported that the DNA probe representing the BTV M6 RNA hybridized with the corresponding RNA segments of 20 out of 20 BTV serotypes tested, indicating that the gene (and gene product) is highly conserved (19). However, unlike similar data obtained with genes representing the viral structural proteins, no hybridization was obtained with the corresponding segments of two epizootic hemorrhagic disease virus serotypes, indicating that NS1 genes are perhaps conserved only by BTV serotypes. If correct, then NS1 antigen would be valuable as a reagent for the diagnosis of BTV infections since antiserum against BTV contained NS1 antibodies (8). To investigate the value of the expressed NS1 protein as a diagnostic reagent, recombinant virus-derived NS1 protein was applied in an ELISA with five available United States BTV antisera. The data obtained indicated that the genetically engineered NS1 antigen was suitable for identifying all the United States BTV serotypes.

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LITERATURE CITED