Characterization and Modification of the Carboxy-Terminal Sequences of Bluetongue Virus Type 10 NS1 Protein in Relation to Tubule Formation and Location of an Antigenic Epitope in the Vicinity of the Carboxy Terminus of the Protein

KATHERINE MONASTYRSKAYA,^{1,2} ERNEST A. GOULD,² AND POLLY ROY^{1,2,3*}

Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, Oxford OX1 3QU,¹ and NERC Institute of Virology and Environmental Microbiology, Oxford OX1 3SR,² United Kingdom, and Department of Public Health Sciences, University of Alabama at Birmingham, Birmingham, Alabama 35294³

Received 9 September 1994/Accepted 23 January 1995

Bluetongue virus produces large numbers of tubules during infection. The tubules are formed from a 552-amino-acid, 64-kDa NS1 protein encoded by the viral double-stranded RNA segment M6. A series of deletion and extension mutants of bluetongue virus serotype 10 NS1 has been generated and expressed in insect cells in order to identify the carboxy-terminal components of the protein which are important for tubule formation. The mutants AcCT5 and AcCT10, lacking 5 and 10 of the carboxy-terminal residues, respectively, were prepared. By analyzing their abilities to form tubules, it was shown that AcCT5 was capable of this function whereas AcCT10 was not, indicating that the last five amino acids are not strongly involved in NS1 tubule formation. Extension mutants including foreign antigenic sequences involving up to 16 amino acids added to the C terminus of NS1 were shown to form tubules, although an extension of 19 amino acids inhibited tubule formation. Analysis of a panel of monoclonal antibodies has established that an NS1 antigenic site is located near the carboxy terminus of the protein. It appears to be exposed on the surface of tubules. The opportunities to develop new vaccines using recombinant NS1 to deliver foreign epitopes are discussed.

Orbiviruses, members of the *Reoviridae* family, are a group of viruses with similar morphological and physicochemical properties. The prototype orbivirus is bluetongue virus (BTV). It is transmitted to vertebrates by *Culicoides* midges and may cause an acute febrile disease in sheep with occasional high mortality. Like other orbiviruses, BTV has a double-stranded RNA (dsRNA) genome consisting of 10 segments (L1 to L3, M4 to M6, and S7 to S10) located in the core of the virus particle. The core contains two major (VP3 and VP7) and three minor (VP1, VP4, and VP6) proteins and is surrounded by an outer shell consisting of two major proteins, VP2 and VP5. In addition to the structural components of the virion, the BTV genome also encodes the nonstructural proteins NS1, NS2, NS3, and the related NS3A.

A distinctive feature of an orbivirus infection is the formation of numerous tubular structures in the cytoplasm of infected cells (23). For BTV serotype 10 (BTV-10), it has been shown that tubules are formed from the 552-amino-acid, 64-kDa NS1 protein encoded by the dsRNA segment M6 (20). Electron microscopic analysis of thin sections of BTV-infected cells has revealed that tubules accumulate in the cytoplasm of infected cells late in infection, particularly in peri- and juxtanuclear locations. It has been also shown by negative staining that they have a helical conformation with a diameter of ca. 68 nm. No definite function has been ascribed to BTV tubules or those of related orbiviruses, although they may be involved in the translocation of virus particles to the cell plasma membrane, where another nonstructural protein, NS3 (NS3A), facilitates the egress of virus particles by causing local lesions in the cell membrane (16).

A cDNA representing BTV-10 M6 dsRNA has been cloned. Expression of the encoded NS1 protein by recombinant baculoviruses results in the synthesis of tubules in insect cells, proving that tubule formation requires no other BTV gene product (27). The expressed tubules have been purified, and their biochemical and biophysical properties have been analyzed. Three-dimensional studies using cryoelectron microscopy and image processing techniques have shown that the tubules are some 52.3 nm in diameter and composed of a coiled ribbon of dimers with ca. 22 dimers per turn. Each NS1 subunit has a diameter of ca. 5.3 nm (11).

The BTV-10 NS1 protein has a low content of charged amino acids but is rich in cysteines, suggesting that it has a highly ordered structure (25). Mutational analyses of the BTV-10 NS1 gene have revealed the importance of certain cysteine residues (C-337 and C-340), as well as the amino and carboxy termini of the protein (22), for tubule formation. In this paper, further effects of deletion and extension mutants of NS1 on tubule formation are reported.

Monoclonal antibodies (MAbs) have been used to investigate the structure and role of NS1 protein in BTV morphogenesis. Several MAbs raised against the NS1 protein of an Australian isolate of BTV-1 have been used to locate NS1 in virus-infected cells (5). Linear epitopes have been identified by using six of these MAbs (8). In the work presented here, a panel of MAbs against the recombinant BTV-10 NS1 protein was produced. Using these MAbs, we identified an antigenic site at the carboxy terminus of the molecule distinct from the two amino-terminal sites described previously (8). On the basis of immunoelectron microscopy, the carboxy-terminal site of NS1 protein appears to be exposed on the surface of tubules. The location of the antigenic region has been confirmed by cloning the last 21 amino acids of BTV-10 NS1 (amino acids 532 to 552) as a glutathione *S*-transferase (GST) fusion protein

* Corresponding author. Mailing address: NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, United Kingdom.

TABLE 1. Oligonucleotide primers used in PCRs

Name	Nucleotide sequence ^a
Bac1	5'-TGATAACCATCTCGCAA-3'
5	5'-GGCCGGATCCCTACTGAGACATGGCCGCA-3'
10	5'-GGCCGGATCCCTAAGCAAGATTAATTACTTCCC-3'
R	5'-GGCCGGATCCCTACTATCTACTCCATCCACATCTG-3'
KRL	5'-GGCCGGATCCCTACAGTCTCTTATACTCCATCCACATC-3'
BLp5	5'-GCGCGGATCCCTATAAGCAAAGGTGATCAGGGGATCATACCCATACTCCATCCACATCTGAG-3'
Rel	5'-GGCCGAATTCTAATACTCCAGCCACATCT-3'
NSS	5'-GCGCGGATCCCTACCCGGGGTCACTAGTACTAGTATACTCCATCCACATCTGAGA-3'
e21	5'-GGCCGGATCCGCGTGTGAAGGCGGG-3'
e44	5'-GGCCGGATCCGAAATGGTTCGATGCATTA-3'

^a *Bam*HI, *Eco*RI, *Spe*I and *Sma*I cloning sites are underlined. CTA antitermination codons are highlighted in boldface. In oligonucleotide primers 5, 10, R, KRL, NSS, BLp5, and Rel, the sequences corresponding to the newly formed carboxy terminus of BTV-10 NS1 are shown in italics. The introduced anticodons for Arg (primer R) and Lys, Arg, and Leu (primer KRL) and the 10-amino-acid peptide (GYDPLITFSL) corresponding to amino acids 131 to 140 of BLV gp51 are shown in boldface italics and double underlined. The primers e21 and e44 represent the coding strand of the NS1 gene (italics) (nucleotides 1628 to 1642 and 1559 to 1577, respectively, counting the first 5'-end nucleotide of BTV-10 M6 dsRNA as 1).

and by demonstrating MAb binding to the expressed product. The addition of foreign epitopes representing bovine leukemia virus (BLV) glycoprotein gp51 on the C terminus of NS1 is reported, demonstrating the potential use of NS1 chimeras in the development of vaccines.

MATERIALS AND METHODS

Viruses and cells. *Spodoptera frugiperda* (Sf) cells were grown in suspension and monolayer cultures at 28°C in TC100 medium supplemented with 10% fetal calf serum. Derivatives of *Autographa californica* nuclear polyhedrosis virus (AcNPV) containing the wild-type BTV-10 NS1 gene (Ac10BTV6) and the NS1 mutants were plaque purified and propagated as described elsewhere (1).

Deletion and extension mutations of BTV-10 NS1 gene and construction of recombinant transfer vectors. PCR was used to construct the NS1 gene deletion mutants, in which either 5 or 10 of the carboxy-terminal amino acids (i.e., residues 548 to 552 or 543 to 552) of the BTV-10 NS1 gene were removed as described previously (22). The forward sense primer Bac1 and the back primers 5 or 10 were used (Table 1). Plasmid pAcCL29.10BTV6 was used as a template. The forward sense primer corresponds to the AcNPV sequence upstream of the NS1 cloning site *Bam*HI. The back primers 5 and 10 include sequences in the opposing strand, corresponding to 5 and 10 amino acids, respectively, before the carboxy terminus of NS1. The two back primers also provide *Bam*HI sites next to the introduced antitermination codons. Extension mutants were obtained by PCR using the Bac1 primer and the back primer R, KRL, or BLp5. The back primers provide *Bam*HI sites and antitermination codons (Table 1) after the inserted sequences.

Vent DNA polymerase (New England Biolabs) was used for the PCR amplifications. The amplified products were treated with proteinase K, phenol extracted, digested with *Bam*HI, and purified by GeneClean as described by the manufacturer (Promega). The mutant sequences were then inserted into the transfer vector pAcYMI as described elsewhere (22), giving the recombinant transfer vectors pAcNS1dCT5, pAcNS1dCT10, pAcNS1eR, pAcNS1eKRL, and pAcNS1eBLp5. The sequences at the insertion sites were confirmed by the dideoxynucleotide technique (26).

For extension mutants pAcNS1eBLp2 and pAcNS1eBLp4, in addition to *Bam*HI sites and antitermination codons, the back primer NSS (Table 1) also provides *Spe*I and *Sma*I sites between the tyrosine Tyr-552 codon TAT and TAG stop codon and corresponds to the C terminus of BTV-10 NS1 (italics in Table 1). The PCRs were performed with the same pAcCL29.10BTV6 template and the forward sense primer Bac1. The amplified product was cloned into pAcYMI, giving the pNS1.SpSm vector, which was subsequently cut with *Spe*I and *Sma*I. Synthetic linkers P2 and P4 (Table 2), with *Spe*I 5' overhangs and encoding peptides representing BLV gp51 (amino acids 177 to 192 and 155 to 167, respectively), were inserted into the cut vector to provide the recombinant transfer vectors pAcNS1eBLp2 and pAcNS1eBLp4, respectively. As a result of these manipulations, the NS1 sequences in the cDNA were extended to encode the following peptides: STPDCAICWEPSPWAPEG* and STNSDWVPSVRSWALG*. Serine and threonine (underlined) residues are encoded by an *Spe*I restriction site (ACTAGT); the glycine residue (underlined) prior to the stop codon (*) is encoded by the second half of the *Sma*I site (CCC*GGG). The peptide sequences (in boldface) correspond to amino acids 177 to 192 and 155 to 167 of BLV gp51, respectively.

Construction of pAcG2T-based recombinant transfer vectors expressing a series of peptides from the carboxy terminus of NS1 as GST fusions. Recombinant baculovirus vectors expressing GST fusion proteins (4) were used to express a panel of small peptides (5, 9, and 12 amino acids) corresponding to the C

terminus of BTV-10 NS1 protein. For this purpose, the pAcG2T vector (4) was used together with synthetic oligonucleotides to provide the required sequences in frame with the termination codon (Table 2). The pAcG2T vector was digested with *Bam*HI and *Eco*RI and ligated with the oligonucleotides. The recombinant transfer vectors pAcG2Te5, pAcG2Te9, and pAcG2Te12 were recovered. A different technique was used to obtain larger GST fusions representing 21 or 44 amino acids of the C terminus of NS1. Sequences encoding the last 21 or 44 amino acids of the BTV-10 NS1 gene were amplified by PCR using the back primer Rel (Table 1), which included an antitermination codon and an *Eco*RI site. The forward sense primers e21 and e44 (Table 1) included *Bam*HI sites to facilitate the insertion into the pAcG2T vector. The PCR products were digested with endonucleases *Bam*HI and *Eco*RI and inserted into *Bam*HI- and *Eco*RI-digested pAcG2T vector as described above, giving the recombinant transfer vectors pAcG2Te21 and pAcG2Te44.

Isolation of recombinant baculoviruses. The lipofection technique (6) was used to cotransfect monolayers of Sf cells with recombinant transfer vectors and *Bsu*361 triple-cut AcNPV DNA (19). The recombinant baculoviruses were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described previously (18). Recombinants were obtained as follows: from pAcNS1dCT5, recombinant AcCT5; from pAcNS1dCT10, recombinant AcCT10; from pAcNS1eR, recombinant AcNS1eR; from pAcNS1eKRL, recombinant AcNS1eKRL; from pAcG2Te5, recombinant AcGSTe5; from pAcG2Te9, recombinant AcGSTe9; from pAcG2Te12, recombinant AcGSTe12; from pAcG2Te21, recombinant AcGSTe21; from pAcG2Te44, recombinant AcGSTe44; from pAcNS1eBLp5, recombinant AcNS1eBLp5; from pAcNS1eBLp2, recombinant AcNS1eBLp2; and from pAcNS1eBLp4, recombinant AcNS1eBLp4.

SDS-PAGE. Sf cells were infected with each recombinant baculovirus at a multiplicity of infection of 5 PFU per cell (7). Cells were harvested at 72 h postinfection, washed with phosphate-buffered saline (PBS), and lysed at 4°C in 50 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.5% Nonidet P-40. Protein dissociation buffer (10% [vol/vol] β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol, 10 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue) was added to each sample, and the mixtures were heated at 100°C for 10 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10 or 20% polyacrylamide gel) and stained with Coomassie brilliant blue.

Purification of tubules. Baculovirus-expressed BTV-10 NS1 tubules were purified as described elsewhere (22, 27). Sf cells were infected in suspension with Ac10BTV6 or the various mutant NS1 expression vectors at a multiplicity of infection of 5. After incubation at 28°C for 72 h, cells were harvested, washed with PBS, resuspended in STE buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.5]) containing 0.5% (vol/vol) Triton X-100, and lysed by homogenization. The lysate was clarified by centrifugation (5 min at 3,000 rpm), and the supernatant was loaded onto a cushion of 3 ml of 40% (wt/vol) sucrose in STE and centrifuged in an SW41 rotor (2 h, 35,000 rpm). The resulting pellet was resuspended in a small volume of STE and loaded onto a 10 to 40% gradient of sucrose in STE and centrifuged in an SW28 rotor (1 h, 20,000 rpm). The pellet and fractions (1.5 ml) of the gradient were collected, and the presence of NS1 was analyzed by SDS-PAGE. Authentic BTV-10 NS1 tubules were purified by a similar method. BHK-21 cell monolayers were infected with BTV-10 virus stock at a multiplicity of infection of 10, incubated at 37°C for 48 h, harvested, and lysed. The cell lysates were clarified and subjected to the one-step centrifugation through a 3-ml 40% sucrose cushion as described above. The resulting pellet was resuspended in STE and used for enzyme-linked immunosorbent assay (ELISA).

Western immunoblot analysis. Proteins from gels were transferred onto a nitrocellulose membrane by blotting procedures. The membrane was then soaked in blocking buffer (5% [wt/vol] skimmed milk–0.05% Tween 20 in PBS) and incubated for 90 min in appropriate antiserum (1:1,000 dilution in blocking buffer). After being washed for 30 min in PBS containing 0.05% Tween 20, the membrane was incubated in blocking buffer containing a 1:1,000 dilution of

TABLE 2. Synthetic linkers used to introduce amino acid sequences

Name	Nucleotide and amino acid sequence of linker ^a
E5.....	<div style="text-align: center;">M W M E Y</div> 5'-GATCTATGTGGATGGAGTATGG-3' 3'-ATACACCTACCTCATACCTTAA-5'
E9.....	<div style="text-align: center;">A M S Q M W M E Y</div> 5'-GATCTGCCATGTCTCAGATGTGGATGGAGTATGG-3' 3'-ACGGTACAGAGTCTACACCTACCTCATACCTTAA-5'
E12.....	<div style="text-align: center;">L A A A M S Q M W M E Y</div> 5'-GATCTCTTGTCTGCGCCATGTCTCAGATGTGGATGGAGTATGG-3' 3'-AGAACGACGCCGTACAGAGTCTACACCTACCTCATACCTTAA-5'
P2.....	<div style="text-align: center;">S T P D C A I C W E P S P P W A P E</div> 5'-CTAGTCCAGACTGTGCTATATGTTGGGAACCTTCCCTCCCTGGGCTCCCGAA-3' 3'-AGGTCTGACACGATATACAACCTTGGGAAGGGGAGGACCCGAGGGCTT-5'
P4.....	<div style="text-align: center;">S T N S D W V P S V R S W A L</div> 5'-CTAGTAACAGTGACTGGGTTCCTCTGTGAGATCATGGCCCTG-3' 3'-ATTGTCACTGACCCAAGGGAGACAGTCTAGTACCCGGGAC-5'

^a The five-amino-acid peptide (E5 linker) represents amino acids 548 to 552 (shown above the nucleotide sequence) of BTV-10 NS1 protein. The 9- and 12-amino-acid peptides (E9 and E12 linkers) represent amino acids 544 to 552 and 541 to 552, respectively. The 5' overhangs to anneal to *Bam*HI- or *Eco*RI-cut DNA are underlined (single line for *Bam*HI, double for *Eco*RI). After ligation of these linkers to cut DNA, the *Bam*HI sites are destroyed. Following the ST sequences, the 16- and 13-amino acid peptides (P2 and P4 linkers) represent amino acids 177 to 192 and 155 to 167, respectively, of the BLV gp51 glycoprotein. The 5' overhangs were designed to anneal to *Spe*I-cut DNA (single underline); the 3' sequences by blunt-end ligation add a further G codon (not shown) before the stop codon.

secondary antibodies (goat anti-rabbit or anti-mouse) conjugated with alkaline phosphatase. Following a second wash, the membrane was treated with an alkaline phosphatase substrate (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium [BCIP] from GIBCO-BRL; prepared in 0.1 M Tris-HCl [pH 8.5]-0.1 M NaCl, 0.05 M MgCl₂ · 6H₂O) for 5 min and then rinsed and dried.

ELISA. The conventional ELISA procedure was performed as described previously (10). MicroELISA plates were coated overnight with an optimal concentration (10 µg per well) of antigen (recombinant or BTV-infected BHK cell NS1 protein) in carbonate coating buffer (15 mM Na₂CO₃, 36 mM NaHCO₃ [pH 9.6]). Plates were washed once with PBS-0.05% Tween 20 and blocked by covering the wells with PBS supplemented with 2% (wt/vol) skimmed milk (50 µl per well). After incubation of the plates at 37°C for 30 min, the wells were washed three times with PBS-Tween, and serial two- or fivefold dilutions of mouse MAb were added for 1 h at 37°C. The plates were then washed again five times with PBS-Tween, and the biotinylated sheep anti-mouse immunoglobulin (Amersham) was added to the plates (50 µl per well, 1:500 in PBS). After 1 h of incubation at 37°C and thorough washing, 100 µl of streptavidin-peroxidase complex (diluted 1:500 in PBS; Amersham) was added to each well, and the plates were incubated at 37°C for 15 min. After the plates were washed five times with PBS-Tween, 100 µl of substrate was added to each well. The substrate was 2,2'-azino-bis-3-ethyl-benz-thiazoline-6-sulfonic acid (ABTS; 0.5 mg/ml) in 25 mM citric acid-25 mM sodium citrate, freshly prepared and supplemented with H₂O₂ (1 µl/ml) just before addition. The reaction was stopped after a maximum of 30 min by adding 100 µl of 3 M NaF per well. The plates were read at a wavelength of 405 nm, using an ELISA reader. In some cases, another method was used. After the first antibody treatment (mouse MAb or rabbit anti-BLV gp51 antibody), wells were covered with alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG; diluted 1:500 in PBS; Sigma), incubated for 1.5 h at 37°C, washed as described above, and detected by adding 100 µl of substrate per well. In this case, the substrate for alkaline phosphatase was 1 mg of *p*-nitrophenyl phosphate disodium (Sigma) per ml in 100 mM glycine-1 mM MgCl₂-1 mM ZnCl₂ (pH 10.4). The reaction was left to develop at 37°C for 30 min and then stopped with 3 N NaOH and read at 405 nm.

Monoclonal and polyclonal antibodies. MAbs were raised against the baculovirus-expressed gradient-purified BTV-10 NS1 tubules by using standard methods (3, 10, 17). All MAbs were detected in the ascitic fluids by ELISA with purified NS1 tubules. The titers of the monoclonal ascitic fluids in ELISA varied between 10³ and 10⁷. The immunoglobulin class and subclass of MAbs were determined by using the isotyping kit for mouse MAbs (Serotec, Kidlington, United Kingdom) as recommended by the manufacturer. MAbs binding linear epitopes were selected by Western blot analysis with both reduced and nonreduced NS1 protein.

The rabbit polyclonal antisera were prepared by the standard protocol. Two rabbits (R745 and R746) were immunized with the purified NS1eBLp4 tubules (1 mg of each). The sera were recovered after a test bleed (21 days after the first injection) and assayed in ELISA with BLV antigen.

Electron microscopy. NS1 tubules were resuspended in water and absorbed onto carbon-coated copper 400-mesh electron microscope grids for 2 to 3 min, washed with water, and negatively stained with 2% (wt/vol) uranyl acetate. Grids were examined with a JEOL 100CX electron microscope under low-dose conditions at 100 kV. For immunogold labelling, the grids were prepared as described above and reacted with anti-NS1 MAb 10B1, and then the NS1-MAB

complexes were labelled with goat anti-mouse antibody coupled to 5-nm gold particles (BioCell Research Laboratories, Cardiff, United Kingdom).

RESULTS

Expression of BTV-10 NS1 deletion mutants by recombinant baculoviruses and effects of these mutations on tubule formation. Previously we reported that the amino and carboxy termini of NS1 are essential for BTV-specified tubule formation. Deletion of the first 10 amino acids as well as the last 20 or 43 amino acids of the protein abrogated tubule formation in insect cells and led to aggregation of NS1 protein (22). To define more precisely which residues of the carboxy-terminal sequence are required, smaller deletion mutants were constructed by removing just 5 or 10 amino acids from the carboxy terminus of NS1 (Materials and Methods). When these mutants, designated AcCT5 and AcCT10, were expressed in insect cells by the recombinant baculoviruses, they produced NS1 proteins which appeared to be similar in size to the wild-type protein (Fig. 1A, lanes 8 and 9). The difference of five amino acid residues between the wild-type NS1 (Fig. 1A, lane 1) and AcCT5 deletion mutant (Fig. 1A, lane 8) is difficult to notice in the SDS-12% polyacrylamide gel stained with Coomassie blue. It is possible that the high expression level of AcCT5 and AcCT10 deletion mutants to some extent concealed the real migration patterns of the truncated NS1 proteins. In this context, it is also noteworthy that the strong hydrophobicity, the high molar ratio of cysteine residues, and the +2 net charge of NS1 influence the migration pattern of NS1 protein in SDS-PAGE: the apparent molecular weight of NS1 is considerably lower than that predicted from its deduced amino acid sequence. As expected, both mutant proteins were recognized by the anti-NS1 polyclonal antiserum as were the mutant NS1 proteins made by AcNT10, AcCT20, and AcCT43 as described earlier (22) (Fig. 1C).

It has been demonstrated that in insect cells, the NS1 protein of BTV forms tubules which can be recovered as a band by gradient centrifugation (22, 27). To examine the tubule-forming capacities of AcCT5 and AcCT10, Sf cells were infected with the appropriate recombinant baculovirus, and 72 h postinfection, cytoplasmic lysates were obtained and centrifuged in sucrose gradients as described in Materials and Methods (Fig. 2). In the case of the AcCT5 mutant (Fig. 2C), the distribution

FIG. 1. Expression of mutant NS1 genes and GST fusion proteins in insect cells by recombinant baculoviruses. Extracts of virus-infected Sf cells were separated by SDS-PAGE (12% polyacrylamide gel) and stained with Coomassie brilliant blue (A and D). The resolved products were electroblotted onto nitrocellulose membranes (B, C, and E) and reacted with MAb 10B1 (B and E) or with a rabbit anti-NS1 polyclonal antiserum (C), and the complexes were identified as described in Materials and Methods. (A to C) Lane 1, purified tubules derived from Ac10BTV6-infected Sf cells; lanes 2 to 9, extracts from uninfected Sf cells (lane 2) or cells infected with Ac10BTV6 (lane 3), AcEHDV2.NS1 (lane 4), AcNT10 (lane 5), AcCT20 (lane 6), AcCT43 (lane 7), AcCT5 (lane 8), or AcCT10 (lane 9). The recombinant baculoviruses AcNT10, AcCT20, and AcCT43 express NS1 deletion mutants lacking the 10 N-terminal amino acids and 20 and 43 C-terminal amino acids, respectively (22). (D and E) Lane 1, purified tubules derived from Ac10BTV6-infected Sf cells; lanes 2 to 7, extracts from uninfected Sf cells (lane 2) or cells infected with AcGSTe5 (lane 3), AcGSTe9 (lane 4), AcGSTe12 (lane 5), AcGSTe21 (lane 6), or AcGSTe44 (lane 7). Positions of protein markers (in kilodaltons) and NS1 are indicated. Open arrowheads indicate the locations of GST-peptide fusion proteins with molecular masses ranging from approximately 26 to 30 kDa.

of NS1 protein in the fractions was similar to that observed for wild-type NS1 (Fig. 2A), whereas for AcCT10, no tubules were identified (Fig. 2D); only aggregated protein similar to that observed previously for the AcCT20 and AcCT43 deletion mutants analyzed previously (22) was detected. Electron microscopy confirmed that similar to results for the wild-type virus (Fig. 3a), NS1 tubules were formed by the AcCT5 mutant (Fig. 3c) and that the AcCT10 deletion made only protein aggregates (Fig. 3d).

Characterization of MAbs raised against the baculovirus-expressed BTV-10 NS1 protein. Fourteen MAbs were prepared and characterized. They were detected by ELISA using gradient-purified Ac10BTV6-expressed NS1 protein or protein obtained from the cell lysates of BTV-10-infected BHK cells as substrates (see Materials and Methods). When isotyped, most MAbs were shown to belong to the IgM group. Very few bound to linear epitopes when tested by Western blotting under either reducing or nonreducing conditions (Table 3).

The MAbs were tested for cross-reactivity with tubules formed by epizootic hemorrhagic disease virus (EHDV). Nine of the MAbs which bound to conformational epitopes of BTV-10 NS1 cross-reacted with the EHDV-2 tubules (Table 3), as expected from the high degree of homology between the EHDV and BTV NS1 proteins (24).

Mapping of the carboxy terminus of BTV-10 NS1 with MAb 10B1. It was demonstrated that MAb 10B1 (an IgG1 type) bound to a linear epitope in BTV-10 NS1 but showed very little

TABLE 3. MAbs

MAb	Isotype	Titer in ELISA	Western blot ^a		Cross-reactivity with EHDV-2 NS1 ^b
			Reduced	Nonreduced	
10B1	IgG1	1×10^7	++++	++++	No
4A7	IgM	5×10^6	—	—	Slight
9F7	IgG1	1×10^3	—	—	Strong
7B4	IgM	1×10^5	+/-	+/-	Strong
6G3	IgM	1×10^5	—	—	Strong
8D2	IgM	1×10^3	+/-	+/-	No
9F12	IgG2a	1×10^3	—	—	Strong
10E1	IgM	5×10^5	—	—	Slight
8D2	IgM	5×10^3	—	+/-	No
9A10	IgM	5×10^6	+/-	+/-	Strong
6E7	IgM	5×10^3	+	+	Strong
3C2	IgM	1×10^5	+/-	+/-	No
7F10	IgM	1×10^5	+	—	No
6C7	IgM	5×10^5	—	—	Strong

^a + + + +, very strong reactivity in a Western blotting reaction; +, less strong reactivity; +/-, rather weak reactivity that took significantly longer to develop than the other position reactions; —, no reaction under the conditions used.

^b Estimated by comparison of MAb titers in ELISA using the same amount of antigen (BTV-10 or EHDV-2 NS1 tubules). Strong cross-reactivity means that the antibody titers in both reactions were similar; slight cross-reactivity was observed when antibody titers in ELISA with BTV-10 NS1 were 3 to 5 times higher than with EHDV NS1; no cross-reactivity was observed when MAb titers in ELISA with BTV NS1 were more than 10 times higher than with EHDV-2 NS1.

FIG. 2. Distribution of wild-type and mutant NS1 following sucrose gradient centrifugation. Infected cell extracts were resolved into fractions (1.5 ml) by sucrose gradient centrifugation (fraction 1, top; fraction 9, bottom; c, control NS1; p, pellet) and analyzed by SDS-PAGE (10% polyacrylamide gel) as described in Materials and Methods. Shown are the products obtained for Ac10BTV6 (A), AcNS1eBLp5 (B), AcCT5 (C), AcCT10 (D), AcNS1eR (E), and AcNS1eKRL (F). Positions of protein markers (in kilodaltons) and NS1 are indicated.

cross-reactivity with EHDV NS1 in ELISA (Table 3). When tested by Western blotting against the wild-type NS1 protein, the various NS1 mutants, or the EHDV NS1, it reacted with wild-type BTV NS1 (Fig. 1B, lanes 1 and 3), with mutant NS1 proteins containing serines in lieu of selected conserved cysteines (data not shown), and with the AcNT10 NS1 deletion mutant (Fig. 1B, lane 5 [22]) but not with EHDV-2 NS1 (Fig. 1B, lane 4) or any of the C-terminus deletion mutants (Fig. 1B, lanes 6 [AcCT20], 7 [AcCT43] [22], 8 [AcCT5], and 9 [AcCT10]). In contrast, the polyclonal anti-NS1 antiserum reacted with all of the BTV NS1 mutants and with EHDV NS1 protein (Fig. 1C).

To confirm that MAb 10B1 bound to amino acids located at the carboxy terminus of BTV NS1, we constructed a series of polypeptides representing fusions between GST and short peptides (5, 9, or 12 residues) representing the C-terminus sequence of NS1. The recombinant transfer vectors pAcG2Te5, pAcG2Te9, and pAcG2Te12 were constructed, and recombinant baculoviruses AcGSTe5, AcGSTe9, and AcGSTe12 were obtained as described in Materials and Methods. Infection of Sf cells with these baculoviruses led to the synthesis of recom-

binant proteins of approximately 26 kDa (Fig. 1D, lanes 3 to 5) representing the expected GST-peptide fusions (4). When the proteins were analyzed by Western blotting using MAb 10B1, no binding was observed (Fig. 1E, lanes 3 to 5). However, when GST fusions with longer carboxy-terminal peptides were constructed (21 and 44 amino acids) and expressed by recombinant baculoviruses (AcGSTe21 and AcGSTe44; Fig. 1D, lanes 6 and 7), the proteins reacted strongly with MAb 10B1 in both Western blot analysis (Fig. 1E, lanes 6 and 7) and ELISA (not shown). When purified BTV wild-type tubules expressed by Ac10BTV6 were reacted with gold particle-tagged MAb 10B1 and visualized by electron microscopy, gold label associated with the tubules was visualized, suggesting that the epitope recognized by the antibody is exposed on the surface of the tubules (Fig. 4).

Synthesis and expression of recombinant NS1 proteins with carboxy-terminal extensions. Since the five terminal amino acids are not required for tubule formation, the effect of additions to the carboxy terminus of NS1 on tubule formation was investigated. The extension mutants of NS1 made by recombinants AcNS1eR and AcNS1eKRL recombinant baculo-

FIG. 3. Electron micrographs of baculovirus-expressed NS1 tubules. (a) Gradient-purified tubules from Ac10BTv6 (wild-type) infection; (b) tubules from AcNS1eBLp5 infection; (c) tubules from AcCT5 infection; (d) NS1 aggregates (indicated by arrows) from AcCT10 infection (an AcNPV particle is also shown); (e and f) gradient-purified tubules from AcNS1eR and AcNS1eKRL infections, respectively. Bars = 100 nm.

FIG. 3—Continued.

FIG. 3—Continued.

FIG. 4. Immunogold labelling of the wild-type NS1 tubules by using MAb 10B1. Purified NS1 tubules absorbed on electron microscope grids were reacted with MAb 10B1 and then with the secondary goat anti-mouse antibody coupled with 5-nm gold particles. The complexes were visualized by electron microscopy.

viruses were propagated as described in Materials and Methods. The AcNS1eR and AcNS1eKRL recombinant baculoviruses expressed mutant NS1 proteins with arginine or with lysine, arginine, and leucine residues in addition to the natural terminus of NS1 (the tyrosine at position 552). Both mutants formed tubules, as judged by sucrose gradient distribution (Fig. 2E and F), similar to those of the wild-type NS1, as determined by electron microscopy (Fig. 3e and f).

Expression of the BLV epitopes in the carboxy terminus of BTV-10 NS1. The various biological functions of the external glycoprotein, gp51, of BLV have recently been mapped by using synthetic peptides (2). In an attempt to insert a foreign peptide in NS1, three different epitopes of BLV were selected. Initially, a 10-amino-acid residue peptide of gp51 was chosen for insertion at the carboxy terminus of BTV-10 NS1 after the last residue (tyrosine in amino acid position 552). The recombinant transfer vector pAcNS1eBLp5 was obtained as described in Materials and Methods and used to generate the recombinant baculovirus AcNS1eBLp5. The mutated NS1 protein containing the 10-amino-acid BLV sequence was synthesized in insect cells, and its ability to form tubules was examined. The distribution of mutated NS1 protein in a sucrose gradient following centrifugation (Fig. 2B) was similar to that observed for the wild-type NS1 protein (Fig. 2A). Electron microscopic examination revealed tubules similar to those formed by the wild-type NS1 (Fig. 3b).

A second sequence representing amino acids 155 to 167 of BLV gp51 was selected for addition to the C terminus of NS1. This sequence is believed to be involved in binding to cellular receptors (2). The recombinant AcNS1eBLp4 was derived and used to express the chimeric NS1. As expected, analysis by

SDS-PAGE showed that the protein was slightly larger (Fig. 5A, lane 2) than the wild-type NS1 protein (Fig. 5A, lane 3). Further, the chimeric protein formed a band in sucrose gradients similar to the wild-type NS1 band. Electron microscopic examination confirmed the presence of tubules with morphologies similar to those of wild-type NS1 (data not shown). In Western blots, the gradient-purified tubules were recognized by the anti-NS1 antiserum (Fig. 5B, lane 2), like the wild-type NS1 tubules (Fig. 5B, lane 3), and uniquely identified by an

FIG. 5. Expression and formation of tubules in insect cells by recombinant baculovirus expressing mutant NS1BLVp4. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue; (B) Western blot using MAb10B1; (C) Western blot using rabbit antiserum raised against a synthetic peptide representing BLV gp51 residues 155 to 164 (2). Lane 1, extract from BLV-infected mammalian cells; lane 2, gradient-purified NS1eBLp4 tubules; lane 3, gradient-purified wild-type NS1 tubules.

FIG. 6. Expression of mutant NS1eBLp2 in insect cells by recombinant baculovirus. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue; (B) Western blot using MAb 10B1; (C) Western blot using rabbit antiserum raised against a synthetic peptide representing BLV gp51 residues 179 to 192. Lane 1, gradient-purified wild-type NS1 tubules; lane 2, extract from uninfected Sf cells; lane 3, extract from AcNS1eBLp2-infected Sf cells. Positions of protein markers (in kilodaltons) are indicated.

antiserum raised against the synthetic peptide representing BLV residues 155 to 164 (Fig. 5C, lane 2) (2).

A third peptide representing amino acid residues 177 to 192 of BLV gp51 protein was subsequently selected for insertion into the carboxy terminus of the NS1 molecule. The sequence has been identified as important in eliciting neutralizing antibody and T-cell responses, as well as involved in binding cell receptors (2). The chimeric recombinant baculovirus, AcNS1eBLp2, was obtained as described in Materials and Methods, and the expressed products were examined by SDS-PAGE (10% polyacrylamide gel). As expected, the chimeric protein (Fig. 6A, lane 3) was slightly larger than the wild-type NS1 protein (Fig. 6A, lane 1). In Western blot analyses, like the wild-type protein (Fig. 6B, lane 1), the mutant was recognized by the anti-NS1 antiserum (Fig. 6B, lane 3). However only the mutant reacted in Western blot analysis with antiserum raised against the synthetic peptide representing residues 179 to 192 of BLV gp51 protein (2) (Fig. 6C, lane 3). Some degraded products of the chimeric protein were present (Fig. 6C, lane 3). When the protein made by the AcNS1eBLp2 mutant was tested for its ability to form tubules, none were identified. The distribution of the mutant NS1 protein in the fractions of sucrose gradient resembled that obtained for the AcCT10 mutant (data not shown). Further, electron microscopic examination of the pellet revealed the presence of NS1 aggregates (data not shown).

To determine whether the chimeric tubules could indeed be used as effective carriers for the presentation of foreign epitopes, the antisera raised against the purified chimeric NS1eBLp4 tubules were tested in ELISA with BLV antigen. As shown in Fig. 7, the antisera recovered from both rabbits gave strong positive reactions with mammalian cells infected with BLV. The titers of the rabbit anti-NS1eBLp4 sera were comparable with the titer of the rabbit antiserum raised against the synthetic peptide representing amino acid residues 155 to 167 of BLV gp51 (2). The anti-NS1 antiserum gave no reaction with the BLV antigen in ELISA, indicating the specificity of the anti-NS1eBLp4 sera. The NS1eBLp5 chimeric tubules were not used for immunization, since the BLV p5 peptide has been shown to be nonreactive with BLV antigen in ELISA or Western blot analysis (2). These data indicate that the NS1 tubules can be used as carrier molecules for presentation of foreign epitopes to be used as immunogens.

FIG. 7. Reactions of antisera raised against NS1eBLp4 chimeric protein with BLV antigen in ELISA. The lysate of mammalian cells infected with BLV was absorbed on microELISA plates, and ELISA was performed with rabbit serum raised against a synthetic peptide representing BLV gp51 residues 155 to 167 (aa155-167), anti-NS1eBLp4 sera from two different rabbits (R745 and R746), and anti-BTV-10 NS1 antiserum, as indicated, using 1:10 to 1:50,000 dilutions of sera.

DISCUSSION

Tubules composed of NS1 protein represent a major constituent of virus-induced products in BTV infections (12). Their function is unknown, although they are often observed to be closely associated with the host cell cytoskeleton as well as with virions and incomplete virus particles within cells (5, 15). The M6 dsRNA is highly conserved between BTV serotypes (9, 14) but much less conserved with the comparable dsRNA species of related orbiviruses such as African horsesickness virus or EHDV (13, 21, 24), although their encoded proteins have homologous sequences.

The study presented here concerned the functional domains of the carboxy terminus of BTV-10 NS1 protein, using deletion, extension, and MAb analysis. Using deletion mutants with 43, 21, 10, and 5 amino acids removed from the carboxy terminus of NS1, it was demonstrated that there is a linear epitope bound by MAb 10B1 located at the C terminus of the protein. When the five-amino-acid peptide representing the last five residues of BTV-10 NS1 was expressed as a fusion with GST, MAb 10B1 failed to bind to the recombinant polypeptide, suggesting that the antibody binding site was larger than five amino acids. Subsequent attempts to express the larger peptides (9, 12, 21, and 44 amino acids originating from the carboxy terminus of NS1) in the same system enabled us to locate the site between residues Ala-531 and Tyr-552. This epitope is specific to BTV (at least BTV-10) and is not present in EHDV-2 NS1, although other epitopes are shared (Table 3). The C-terminal sequence of BTV-10 NS1 is APACGGGEV INLAAAMSQMWMEY (amino acids 530 to 552 [20]); for EHDV-2, the corresponding sequence is APECDGAVM NIAQAMANMWDN (amino acids 531 to 552; the differences from the BTV-10 sequence are underlined [24]). In this context, it is interesting that the NS1 proteins of other BTV serotypes contain an arginine residue instead of an alanine at amino acid position 543 (9, 14). Experiments are in progress to

test whether MAbs 10B1 binds to the NS1 proteins of these other BTV serotypes.

Comparison of BTV-10 and EHDV-2 NS1 sequences has revealed that although overall the amino acid homology is no more than 65%, there are some regions, predominantly hydrophobic, where the amino acid similarity reaches more than 80% (24). Also it has been demonstrated by negative staining that although structurally similar, the BTV- and EHDV-derived tubules have different diameters (68 nm for BTV and 54 nm for EHDV). The sequence similarity is in keeping with the evidence that certain MAbs raised to BTV-10 NS1 cross-reacted in ELISA with EHDV-2 NS1 tubules synthesized in insect cells by recombinant baculoviruses (Table 3).

In our previous work (22), we showed that deletion of either 43 or 20 amino acids from the carboxy terminus of NS1 abrogated tubule formation. A deletion of the 10 carboxy-terminal amino acids also abrogated this function. However, deletion of the terminal five amino acids did not affect tubule formation in insect cells. Since the five terminal amino acids were not required, extensions to the carboxy terminus were analyzed, and it was found that up to 16 additional amino acids, including antigenic sequences originating from BLV gp51, could be added without impairing the ability of the derived NS1 to form tubules. It was also shown that the antisera raised against the NS1-BLV chimeric protein NS1eBLP4 recognize BLV antigen in ELISA. However, the addition of slightly larger sequences resulted in the abrogation of tubule formation. It is worth noting that the larger BLV sequence introduced included five proline residues. Whether this inhibition of tubule formation is size or sequence dependent is not known. Other peptides with different sequences need to be investigated to confirm whether the inhibition is conformation or size dependent. The fact that the addition of up to 16 amino acids at the carboxy terminus of BTV-10 NS1 does not affect tubule formation opens up the possibility of using recombinant BTV-10 NS1 proteins for the development of new vaccines and as a carrier molecule for delivery of foreign epitopes as immunogens.

ACKNOWLEDGMENTS

We are grateful to David H. L. Bishop for his continuous support and discussion of this research and for his help with the preparation of the manuscript. We also thank T. F. Booth and S. Moss for electron microscopy, M. Mikhailov for completing the gel analysis presented in Fig. 5, and C. Hatton for photographic work. We are grateful to A. Burny and D. Portetelle (Faculty of Agronomy, Gembloux, Belgium) for providing us with BLV reagents.

The work was funded by MRC grant G9020032CA and by NIH grants A126879 and 5R01 AI36531-2.

REFERENCES

- Brown, M., and P. Faulkner. 1977. A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *J. Gen. Virol.* **36**:361–364.
- Callebaut, I., V. Voneche, A. Mager, O. Fumiere, V. Krchnak, M. Merza, J. Zavada, M. Mammerickx, A. Burny, and D. Portetelle. 1993. Mapping of B-neutralizing and T-helper cell epitopes on the bovine leukemia virus external glycoprotein gp51. *J. Virol.* **67**:5321–5327.
- Cammack, N., and E. A. Gould. 1986. Topographical analysis of epitope relationships on the envelope glycoprotein of yellow fever 17D vaccine and the wild type Asibi parent virus. *Virology* **150**:333–341.
- Davies, A. H., J. B. M. Jowett, and I. M. Jones. 1993. Recombinant baculovirus vectors expressing glutathione-S-transferase fusion proteins. *BioTechnology* **11**:933–936.
- Eaton, B. T., A. D. Hyatt, and J. R. White. 1988. Localization of the non-structural protein NS1 in bluetongue virus infected cells and its presence in virus particles. *Virology* **163**:527–537.
- Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, H. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413–7417.
- French, T. J., and P. Roy. 1990. Synthesis of bluetongue virus (BTV) core-like particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. *J. Virol.* **64**:1530–1536.
- Gould, A. R., J. S. Martyn, and L. Stevenson. 1994. Expression of the non-structural protein NS1 of bluetongue virus in bacteria and yeast: identification of two antigenic sites at the amino terminus. *Virus Res.* **31**:291–303.
- Gould, A. R., L. I. Pritchard, and M. D. Tavarria. 1988. Nucleotide and deduced amino acid sequences of the non-structural protein, NS1, of Australian and South African bluetongue virus serotype 1. *Virus Res.* **11**:97–107.
- Gould, E. A., A. Buckley, and N. Cammack. 1985. Use of the biotin-streptavidin interaction to improve flavivirus detection by immunofluorescence and ELISA tests. *J. Virol. Methods* **11**:41–48.
- Hewat, E. A., T. F. Booth, R. H. Wade, and P. Roy. 1992. 3-D reconstruction of bluetongue virus tubules using cryoelectron microscopy. *J. Struct. Biol.* **108**:35–48.
- Huismans, H. 1979. Protein synthesis in bluetongue-infected cells. *Virology* **92**:385–396.
- Huismans, H., and M. Cloete. 1987. A comparison of different cloned bluetongue virus genome segments as probes for the detection of virus-specified RNA. *Virology* **158**:373–380.
- Hwang, G.-Y., J.-F. Chiou, Y.-Y. Yang, and J. K.-K. Li. 1993. High-sequence conservation among the United States bluetongue viruses cognate M2 genes which encode the nonstructural NS1 tubule protein. *Virology* **192**:321–327.
- Hyatt, A. D., and B. T. Eaton. 1988. Ultrastructural distribution of the major capsid proteins within bluetongue virus and infected cells. *J. Gen. Virol.* **69**:805–815.
- Hyatt, A. D., Y. Zhao, and P. Roy. 1993. Release of bluetongue virus-like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virology* **193**:592–603.
- Kimura-Kuroda, J., and K. Yasui. 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus using monoclonal antibodies. *J. Virol.* **45**:124–132.
- King, L. A., and R. D. Possee. 1992. The baculovirus expression system. A laboratory guide. Chapman & Hall, London.
- Kitts, P. A., and R. D. Possee. 1993. A method for producing recombinant baculovirus expression vectors at high efficiency. *BioTechniques* **14**:810–817.
- Lee, J., and P. Roy. 1987. Complete sequence of the NS1 gene (M6 RNA) of US bluetongue virus serotype 10. *Nucleic Acids Res.* **15**:7207.
- Mizukoshi, N., K. Sakamoto, A. Iwata, T. Tsuchiya, S. Ueda, T. Watanabe, M. Kamada, and A. Fukusho. 1993. The complete sequence of African horsesickness virus serotype 4 (vaccinia strain) RNA segment 5 and its predicted polypeptide compared with NS1 of bluetongue virus. *J. Gen. Virol.* **73**:2425–2428.
- Monastyrskaya, K., T. F. Booth, L. Nel, and P. Roy. 1994. Mutation of either of two cysteine residues or deletion of the amino or carboxy terminus of nonstructural protein NS1 of bluetongue virus abrogates virus-specified tubule formation in insect cells. *J. Virol.* **68**:2169–2178.
- Murphy, F. A., E. C. Borden, R. E. Slope, and A. Harrison. 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus—a new taxonomic group. *Electron microscopic studies.* *J. Gen. Virol.* **13**:273–288.
- Nel, L., L. A. Picard, and H. Huismans. 1990. A characterization of the nonstructural protein from which virus-specified tubules in epizootic haemorrhagic disease virus-infected cells are composed. *Virus Res.* **18**:219–230.
- Roy, P., J. J. A. Marshall, and T. J. French. 1990. Structure of the bluetongue virus genome and its encoded proteins. *Curr. Top. Microbiol. Immunol.* **162**:43–87.
- Sanger, J. A., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- Urakawa, T., and P. Roy. 1988. Bluetongue virus tubules made in insect cells by recombinant baculovirus: expression of NS1 gene of bluetongue virus serotype 10. *J. Virol.* **62**:3919–3927.