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

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Virus-specific tubules are characteristic of orbivirus infections and are likely to play an important role in virus morphogenesis. It has been shown that for bluetongue virus (BTV), the prototype orbivirus in the family *Reoviridae*, the virus-encoded NS1 protein forms tubules in insect cells when the BTV segment M6 gene is expressed by using a baculovirus vector. To understand the function of NS1 tubules and to identify the sequences involved in their polymerization, a series of mutant NS1 genes was generated and expressed in insect cell cultures by using baculovirus vectors. Three of the mutants were deletion mutants. One (AcNS1.dNT10) lacked 10 of the amino-terminal amino acids, and the other two mutants (AcNS1.dCT20 and AcNS1.dCT43) lacked 20 or 43 of the carboxy-terminal amino acids. In addition, site-directed mutants were constructed in which various single cysteines or pairs of cysteines were changed to serines. The ability of each mutant protein to form tubules was investigated. None of the deletion mutants formed tubules. The constructs in which the cysteines at amino acid positions 337 and/or 340 were replaced by serines (e.g., AcNS1.C337S,C340S) also did not form tubules. Instead, the NS1 protein of these and the deletion mutants made ribbon-like structures which formed large aggregates. Mutations involving six other cysteines (i.e., AcNS1.C37S,C43S, AcNS1.C462S,C465S, AcNS1.C104S, and AcNS1.C364S) produced tubules. The results show that both the amino and carboxy termini of the NS1 protein molecule and the cysteines at residues 337 and 340 are essential for tubule formation.

Microtubules, actin, and intermediate filaments are common structural features of eukaryotic cells. Their various functions have been analyzed extensively (1, 25, 29, 32). Unlike cellular microtubules, virus-derived tubules are a rarity, and where they occur, their function is not known (4, 27, 31). Orbiviruses, members of the family *Reoviridae*, produce large numbers of tubular structures during infections (22). For the prototype orbivirus, bluetongue virus (BTV), it has been shown that tubules are formed from a 552-amino-acid, 64-kDa NS1 protein encoded by the viral double-stranded RNA genome segment M6 (30). The protein is one of three nonstructural proteins made by BTV (27). Electron microscopic analyses of thin sections of BTV-infected cells have revealed that tubules accumulate late in infection particularly in peri- or juxtanuclear locations. Also it has been shown by electron microscopy of the negatively stained tubules that they have a helical conformation with a diameter of ca. 68 nm (9). So far, no function has been ascribed to BTV tubules or those of other orbiviruses, although they may be involved in the translocation of virus particles to the cell plasma membrane, where another nonstructural protein, NS3, facilitates the egress of virus particles from infected cells by causing local lesions in the cell membrane (12).

To gain an insight into the possible role of tubules in BTV

replication and to investigate the structure-function relationships of the NS1 protein, cDNA representing BTV serotype 10 (BTV-10) double-stranded RNA segment M6 has been cloned and expressed in a baculovirus vector (30). Expression of the encoded NS1 protein resulted in the synthesis of tubules in insect cells, proving that tubule formation requires no other viral gene product (30). The expressed tubules have been purified, and their biochemical and biophysical properties have been analyzed (7, 20). Three-dimensional studies of tubules using cryoelectron microscopy and image processing techniques have shown that they are some 52.3 nm in diameter and composed of a coiled ribbon of dimers with ca. 22 dimers per helix turn (7). Each NS1 subunit appears to have a diameter of ca. 5.3 nm (7).

The BTV-10 NS1 protein has a low content of charged amino acids but is rich in cysteines (10, 17, 27), suggesting that it has a highly ordered structure. Also, there are several clearly defined hydrophobic regions, particularly in the carboxyl-terminal half of the protein (17, 27). Interestingly, six of the cysteines are clustered in pairs with two or five amino acids in between, suggesting that they may form double cysteine bridges with other cysteines. So far, no evidence has been obtained for intermolecular bridges, indicating that probably only intramolecular disulfide bonds occur. To determine the nature of the NS1 interactions involved in tubule formation, both deletion and site-directed mutants of NS1 have been prepared and analyzed following expression in baculovirus vectors. Both biochemical and electron microscopic data reveal that certain cysteines as well as both termini of the NS1 protein are important for tubule formation.

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MATERIALS AND METHODS

Viruses and cells. *Spodoptera frugiperda* cells were grown in suspension or monolayer cultures at 28°C in TC100 medium supplemented with 10% fetal calf serum (2). Derivatives of *Autographa californica* nuclear polyhedrosis virus (AcNPV) containing the wild-type BTV-10 NS1 gene (Ac10BTV6) and the various NS1 mutants were plaque purified and propagated as described elsewhere (2).

Site-directed mutagenesis and construction of recombinant transfer vectors. Site-directed mutagenesis was initially used to change pairs of the conserved cysteine residues of the BTV-10 NS1 protein to serines. Mutant plasmids were derived to prepare expression vectors and to introduce additional changes. To facilitate the preparation of these mutants, the wild-type BTV-10 NS1 gene was recovered from the plasmid transfer vector pAcYM1.10BTV6 (30) by excision with *Bam*HI and subcloned into the *Bam*HI site of pAcCL29, a pAcYM1-based transfer vector with single-stranded capability (19), giving the recombinant transfer vector pAcCL29.10BTV6. Mutants were obtained according to published methods (16) and by using oligonucleotides. The first oligonucleotide, C37S,C43S, represented the complement of BTV-10 NS1 nucleotides 138 to 168 with serine anticodons in lieu of cysteine anticodons (counting the 5'-end nucleotide of BTV-10 segment 6 double-stranded RNA as residue 1). The sequence of this oligonucleotide was 5'-TGACACTCATTCCATTAAATAGACTATTCC-3'. The underlined triplets represent serine anticodons. Similarly, the sequence of the second oligonucleotide, C337S,C340S, representing the complement of BTV-10 NS1 nucleotides 1035 to 1061, was 5'-TCAAGTAGCTAAGTTGACTTGTATGT-3'. The sequence of the third oligonucleotide, C462S,C465S, representing the complement of BTV-10 NS1 nucleotides 1410 to 1436, was 5'-ATCCGTAACCTTACAAACTGAATAGC-3'. The other four oligonucleotides were used to introduce single cysteine-to-serine mutations. The sequence of oligonucleotide C337S, representing the complement of BTV-10 NS1 nucleotides 1034 to 1054, was 5'-GCAAAGTTGACTTGTATGTT-3'. The sequence of oligonucleotide C340S, representing the complement of BTV-10 NS1 nucleotides 1041 to 1062, was 5'-TTCAAGTAGCTAAGTTGACAT-3'. The sequence of oligonucleotide C104S, representing the complement of BTV-10 NS1 nucleotides 333 to 353, was 5'-GCGCTCCGCTTCGCTTCATC-3'. The sequence of oligonucleotide C364S, representing the complement of BTV-10 NS1 nucleotides 1116 to 1134, was 5'-CGGCGAGCTCCAGTTAA-3'. The mutated sequences in the recombinant plasmids that were initially derived (pNS1.C37S,C43S, pNS1.C337S,C340S, and pNS1.C462S,C465S), as well as in pNS1.C337S, pNS1.C340S, pNS1.C104S, and pNS1.C364S, were identified by sequence analyses (28). Subsequently, pNS1.C37S,C43S was mutated with oligonucleotide C337S,C340S to give the transfer vector pNS1.C37S,C43S,C337S,C340S, which has four mutated cysteines. This plasmid was then used to make a further mutant transfer vector, using oligonucleotide C462S,C465S, so that six cysteines were mutated (pNS1.C37S,C43S,C337S,C340S,C462S,C465S). Similarly, pNS1.C462S,C465S was mutated to give the transfer vector pNS1.C337S,C340S,C462S,C465S, with the indicated combination of four mutated cysteines.

Deletion mutations of the BTV-10 NS1 gene and construction of recombinant transfer vectors. To construct NS1 deletion mutants, the 10 amino-terminal amino acids (residues 1 to 10) or 20 or 43 of the carboxy-terminal amino acids (i.e., residues 532 to 552 or 509 to 552) of the BTV-10 NS1 gene were deleted by PCR. Plasmid pAcCL29.10BTV6 was used for

the mutageneses. The N-terminal deletion was constructed by using the forward-sense primer (5'-CGCGGATCCCAATATGAGTGGGGATTACGCAAAT-3') and the back primer (5'-CGCACAGAATCTAGCGC 3'). The forward primer contains a *Bam*HI site (underlined) upstream of an ATG codon (in boldface) representing a methionine substitution of the 10th amino acid of NS1. The remaining sequence, in italics, represents the downstream NS1 coding sequence. The back primer corresponds to a region of the AcNPV genome that is some 60 nucleotides downstream of the NS1 insertion site and represents the sequence in the opposing DNA strand. Following *Bam*HI digestion of the PCR product, the mutant gene was ligated into pAcYM1, giving the recombinant transfer vector pNS1dNT10. The two C-terminal deletions were constructed by using the forward sense primer (5'-TGATAACCCTCTCGCAA-3') and the back primer (5'-CGCGGATCCCTCAGCAGGCGCAGCGAA-3') or (5'-CGCGGATCCCTTAAACATTGTAGCATAAGCTC-3'). In this case, the forward primer corresponds to the AcNPV sequence upstream of the NS1 gene. The back primers correspond to sequences (in italics) in the opposing strand and corresponding to 20 and 43 amino acids, respectively, before the carboxy terminus of NS1. The two back primers have *Bam*HI sites (underlined) next to introduced antitermination codons (in boldface). The amplified products were treated with proteinase K, extracted with phenol, digested with *Bam*HI and purified by GeneClean as described by the manufacturer (Promega). The mutant sequences were then inserted into pAcYM1 as described elsewhere (30), giving the recombinant transfer vectors pNS1dCT20 and pNS1dCT43. The sequences at the insertion sites were confirmed by the dideoxynucleotide technique (28).

Isolation of recombinant baculoviruses. By using lipofection (5), monolayers of *S. frugiperda* insect cells were cotransfected with recombinant transfer vectors containing mutated NS1 and *Bsu*36I triple-cut AcNPV DNA as described by Kitts and Possee (15). The recombinant baculoviruses generated as the result of homologous recombination were selected by their LacZ⁻ phenotypes and plaque purified. From pNS1.C37S,C43S, the recombinant AcNS1.C37S,C43S was obtained; likewise, from pNS1.C337S,C340S the recombinant AcNS1.C337S,C340S, from pNS1.C462S,C465S the recombinant AcNS1.C462S,C465S, from pNS1.C37S,C43S,C337S,C340S the recombinant AcNS1.C37S,C43S,C337S,C340S, from pNS1.C37S,C43S,C337S,C340S,C462S,C465S the recombinant AcNS1.C37S,C43S,C337S,C340S,C462S,C465S, from pNS1.C337S,C340S,C462S,C465S the recombinant AcNS1.C337S,C340S,C462S,C465S, from pNS1.C337S the recombinant AcNS1.C337S, from pNS1.C340S the recombinant AcNS1.C340S, from pNS1.C104S the recombinant AcNS1.C104S, and from pNS1.C364S the recombinant AcNS1.C364S were recovered. Also, from pNS1dNT10 the recombinant AcNT10, from pNS1dCT20 the recombinant AcCT20, and from pNS1dCT43 the recombinant AcCT43 were obtained.

Protein analysis by SDS-PAGE. *S. frugiperda* cells were infected with each recombinant baculovirus at a multiplicity of infection of 5 PFU per cell (6). 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] mercaptoethanol, 10% [wt/vol] sodium dodecyl sulfate [SDS], 25% [vol/vol] 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) and stained with Kenacid blue R (6).

Purification of tubules. *S. frugiperda* cells were grown in

suspension culture and infected with Ac10BTV6 or the various mutant NS1 expression vectors, using a multiplicity of infection of 5. After incubation at 28°C for 72 h, the cells were harvested, washed with PBS, and 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. Cells were disrupted by homogenization, and the nuclei and cell debris were pelleted (1,500 × g, 5 min). The supernatant was loaded over a cushion of 3 ml of 40% (wt/vol) sucrose in STE buffer and centrifuged in an SW41 rotor (2 h, 35,000 rpm). The resulting pellet was resuspended in a small volume of STE buffer, loaded onto a 10 to 40% gradient of sucrose in STE buffer, and then 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.

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% [vol/vol] Tween 20 in PBS) and then incubated for 90 min in rabbit anti-NS1 antiserum (1:1,000 dilution in blocking buffer). After a 30-min wash in PBS containing 0.05% Tween 20, the filter was incubated in blocking buffer containing a 1:1,000 dilution of goat anti-rabbit antiserum conjugated with alkaline phosphatase. Following a second wash, the filter was treated with an alkaline phosphatase substrate (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate tetrazolium [salt] [GIBCO-BRI], prepared in 0.1 M Tris-HCl [pH 8.5]–0.1 M NaCl–0.05 MgCl·6H₂O) for 5 min, rinsed, and dried.

Electron microscopy. NS1 tubules resuspended in water were adsorbed onto carbon-coated copper 400-mesh electron microscopy 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. Cells infected with recombinant baculoviruses expressing NS1 were fixed in 0.1% paraformaldehyde and embedded in LR white resin for sectioning. Sections on grids were labelled by using rabbit antiserum raised against BTV-10 grown in BHK cells, and immune complexes were detected with goat anti-rabbit antibodies coupled to 10-nm gold particles (BioCell Research Laboratories, Cardiff, United Kingdom).

RESULTS

Synthesis of recombinant baculoviruses expressing NS1 protein with serine residues in lieu of selected pairs of cysteines. The NS1 protein of BTV-10 contains 16 cysteine residues (17) (Fig. 1). Thirteen are conserved between BTV-10 NS1 and the NS1 protein of the related orbivirus, epizootic hemorrhagic disease virus type 2 (23). Nine are conserved between BTV-10 NS1 and that of African horsesickness virus (21, 24). Interestingly, six of the cysteines conserved between all three viruses are arranged in pairs (Fig. 1) with two or five amino acids in between. These observations raise the question of whether any or all of these conserved cysteines are involved in the polymerization of NS1 into tubular structures. To address this question, initially pairs of cysteines were mutated into serines on the basis that two changes may give a more pronounced phenotype than a single change, allowing the option to study single mutations subsequently. Using the single-strand capacity of pAcCL29 (19), we used synthetic oligonucleotides to prepare NS1 mutants by the method described by Kunkel et al. (16). Each primer was designed so that the neighboring cysteines would both be changed to serines. These primers were used separately or consecutively as

FIG. 1. Diagram of BTV-10 NS1 protein and derived deletion mutants. (a) Schematic of the BTV-10 NS1 protein in which the various cysteine residues are represented by vertical bars. Thick and thin bars represent cysteines conserved between the NS1 proteins of BTV-10 and epizootic hemorrhagic disease virus type 2; thick bars represent the cysteines which were changed to serines in the mutants prepared in this study. The amino acid positions of mutated cysteine residues are indicated. Dotted bars marked with asterisks represent nonconserved cysteine residues. (b) Schematic of the NT10 deletion. (c) Schematic of the CT20 deletion. (d) Schematic of the CT43 deletion.

described in Materials and Methods to generate the recombinant transfer vectors containing two to six mutations. Subsequently, four single-point mutations, each containing serines in lieu of cysteines, were generated by similar procedures.

Each of the recombinant transfer vectors was then used to generate recombinant baculoviruses (see Materials and Methods). As illustrated in Fig. 2A (lanes 4 to 9) and C (lanes 3 to 6), insect cells infected with each recombinant baculovirus synthesized levels of NS1 protein comparable to the high level made by the nonmutant Ac10BTV6 (Fig. 2A, lane 3). Also shown in Fig. 2A and C are the bands obtained with gradient-purified wild-type BTV NS1 tubules (lane 1) as well as uninfected *S. frugiperda* cell extracts (lane 2). All of the mutant proteins were found to be comparable in size to that of wild-type NS1 protein made by Ac10BTV6 (Fig. 2A). The identities of the various mutated NS1 proteins were confirmed by Western blot analysis (Fig. 2B and D) using anti-NS1 antiserum raised against NS1 tubules (30).

Tubule-forming capacity of mutant NS1 proteins. When the NS1 protein of BTV is expressed in insect cells, it forms tubules that can be isolated by sucrose gradient centrifugation (30). Similar procedures were therefore used to analyze the mutant NS1 proteins. Insect cells infected with each recombi-

FIG. 2. Expression of mutated BTV-10 NS1 genes in insect cells by recombinant baculoviruses. Extracts of the virus infected *S. frugiperda* cells were separated by SDS-PAGE and stained with Kenacid blue R (A and C). The resolved products were electroblotted onto nitrocellulose membranes (B and D) and reacted with rabbit anti-NS1 serum, and the complexes were identified as described in Materials and Methods. (A and B) Lanes: 1, purified tubules derived from Ac10BTV6-infected *S. frugiperda* cells; 2 to 12, extracts from uninfected *S. frugiperda* cells (lane 2) and from Ac10BTV6-infected (lane 3), AcNS1.C37S,C43S-infected (lane 4), AcNS1.C337S,C340S-infected (lane 5), AcNS1.C462S,C465S-infected (lane 6), AcNS1.C37S,C43S,C337S,C340S-infected (lane 7), AcNS1.C337S,C340S,C462S,C465S-infected (lane 8), AcNS1.C37S,C43S,C337S,C340S,C462S,C465S-infected (lane 9), AcCT20-infected (lane 10), AcCT43-infected (lane 11), and AcNT10-infected (lane 12) cells. (C) Lanes: 1, purified wild-type tubules; 2 to 6, extracts from uninfected *S. frugiperda* cells (lane 2) and from AcNS1.C104S-infected (lane 3), AcNS1.C337S-infected (lane 4), AcNS1.C340S-infected (lane 5), and AcNS1.C364S-infected (lane 6) cells. (D) Lanes: 1, gradient-purified NS1 tubules; 2 to 5, extracts from AcNS1.C104S-infected (lane 2), AcNS1.C337S-infected (lane 3), AcNS1.C340S-infected (lane 4), and AcNS1.C364S-infected (lane 5) cells. The positions of protein markers (in kilodaltons) and NS1 are indicated.

nant baculovirus were lysed, and the cytoplasmic extracts were centrifuged in gradients of sucrose as described in Materials and Methods. To provide a positive control, NS1 tubules representing the wild-type gene were purified similarly. As shown in Fig. 3A, analyses by SDS-PAGE of sucrose gradient fractions of the resolved wild-type NS1 products demonstrated the distribution of tubules in a broad peak, predominantly in fractions 3 to 5. NS1 protein was also found in the pellet (Fig. 3A, lane P).

Of the six mutant NS1 proteins with two or more cysteine residues changed to serines, only two formed tubules similar to those made by wild-type NS1. Shown in Fig. 3B is the gradient distribution obtained for AcNS1.C37S,C43S. The result obtained for AcNS1.C462S,C465S was similar (data not shown). By contrast, the four other site-directed multiple mutant NS1 proteins, all of which possessed the C337S,C340S change, did not form tubules (Fig. 3D shows the result for AcNS1.C337S,C340S,C462S,C465S). Identical results were obtained for the

other three mutants (data not shown). For these four mutants, NS1 protein was found only in the pellet at the bottom of the gradient. The distribution of NS1 in the gradient suggested that in each case, the mutant NS1 protein aggregated instead of forming the normal, highly ordered tubular structures.

To confirm the physical status of the NS1 protein, fractions of each gradient containing NS1 tubules were examined by electron microscopy. Figure 4a shows the tubules formed by wild-type NS1, while Fig. 4b shows that similar tubules were also formed by AcNS1.C37S,C43S. The result obtained for AcNS1.C462S,C465S was similar (Fig. 4c). Figure 4d shows the material from the pellet of the gradient-resolved AcNS1.C337S,C340S extract. The materials recovered from the pellets of extracts made from AcNS1.C337S,C340S,C462S,C465S, AcNS1.C37S,C43S,C337S,C340S, and AcNS1.C37S,C43S,C337S,C340S,C462S,C465S infections were similar (data not shown). No tubules were identified in these preparations; only ribbon-like structures were found (Fig. 4d). The pellets of the

FIG. 3. Distribution of wild-type and mutant NS1 following sucrose gradient centrifugation. Infected cell extracts were resolved by sucrose gradient centrifugation. The 10 1.5-ml fractions obtained (1, top; 10, bottom; P, pellet; c, control NS1) were analyzed by SDS-PAGE as described in Materials and Methods. Shown are the distributions of products obtained for Ac10BTV6 (A), AcNS1.C37S,C43S (B), AcCT20 (C), AcNS1.C337S,C340S,C462S,C465S (D), AcNS1.C337S (E), and AcNS1.C340S (F). The positions of protein markers (in kilodaltons) and NS1 are indicated.

wild-type preparation, or those of AcNS1.C37S,C43S and AcNS1.C462S,C465S, contained tubules and protein aggregates (data not shown).

To determine whether the purification procedure caused the disruption of tubules, sections of infected cells were examined by immunogold electron microscopy to identify the presence of NS1 protein and structures involving NS1 (Fig. 5). The mutant AcNS1.C37S,C43S produced characteristic tubules which were labelled in transverse and longitudinal orientations (Fig. 5a).

In all cases in which no tubules were identified by gradient

centrifugation, aggregates of material and no tubules were observed in the sectioned cells (Fig. 5b). Thus, the mutant NS1.C37S,C340S produced aggregates of NS1 protein only in the cell cytoplasm, aggregates that appeared to be associated with intermediate filaments or other cytoskeleton components (Fig. 5b). Similar structures were seen with the other mutants which did not form tubules (data not shown).

Synthesis and expression of recombinant NS1 proteins with point mutations of single cysteine residues and their tubule-forming capacities. The results described above indicated that the cysteines at amino acid positions 337 and 340 were

FIG. 4. Electron micrographs of baculovirus-expressed mutant NS1 tubules. (a) Gradient-purified tubules from an Ac10BTv6 infection; (b and c) gradient-purified tubules from AcNS1.C37S,C43S and AcNS1.C462S,C465S infections, respectively; (d) ribbon-like structures (arrows) found in the gradient pellets of the AcNS1.C337S,C340S mutant; (e and f) NS1 aggregates found in the gradient pellets of the AcNS1.C337S and AcNS1.C340S mutants.

FIG. 4—Continued.

required for tubule formation. To determine whether both amino acids were needed for tubule formation, expression vectors with single NS1 mutations (AcNS1.C337S and AcNS1.C340S) were prepared. Analyses of cells infected with either mutant failed to identify tubules in lysates resolved by gradient centrifugation; only pelleted materials containing NS1 were recovered (Fig. 3E and F). Electron microscopic examination of these pellets revealed the aggregates of ribbons similar to those shown in Fig. 4d (Fig. 4e and f). These results indicated that substitution of either of these two cysteines abrogated tubule formation.

In contrast to most of the other conserved cysteines of BTV-10 NS1, C-337 and C-340 are located in a hydrophilic region of the protein. Since another conserved cysteine, C-104, is also located in a hydrophilic region of NS1, the possibility that it was also required for tubule formation was examined. A mutant virus (AcNS1.C104S) was therefore prepared and examined for its ability to form tubules. The distribution of tubules in sucrose gradients made by this mutant NS1 was similar to that obtained with the wild-type NS1 (data not shown). Also, tubules resembling those shown in Fig. 4b were observed by electron microscopy. In view of this observation, it was concluded that the C-104 residue was not required for tubule formation. The requirement for the cysteine at position 364 for tubule formation was similarly investigated. A recombinant virus (AcNS1.C364S) was prepared, and tubule synthesis was investigated as described above. Gradient and electron microscopic analyses identified the formation of tubules re-

sembling those made by Ac10BTV6 (data not shown). These data indicated that this cysteine is also not critical for tubule formation.

Deletion of the amino or carboxy terminus of the BTV NS1 protein abrogates tubule formation. The BTV-10 NS1 protein molecule has a number of hydrophobic regions throughout the molecule, particularly in the carboxyl-terminal half (17, 27). The amino terminus, on the other hand, is only slightly hydrophobic. The roles played by these regions in the formation of tubules are not known. We therefore constructed deletion mutants, one involving the removal of 10 amino acids from the amino terminus and the others involving removal of 20 or 43 amino acids from the carboxy terminus (Materials and Methods; Fig. 1). The expressed NS1 proteins of all three mutants were slightly smaller than the wild-type protein, as illustrated in Fig. 2A (lanes 10 to 12). The mutant proteins were recognized by anti-NS1 serum, as shown in Fig. 2B. When the recombinants were examined by sucrose gradient centrifugation for the ability to form tubules, no tubules were identified (Fig. 3C shows the results for AcCT20). All of the NS1 protein was recovered in the pellets. Examination by electron microscopy revealed only ribbon-like aggregates of material in the pellets (Fig. 6).

In summary, the three deletion mutants failed to form tubules when expressed in insect cells by baculovirus vectors, an indication that regions of both the amino and carboxy termini are important for tubule formation.

FIG. 5. NS1 distribution in cells analyzed by immunogold analyses. (a) Section of the cytoplasm of an *S. frugiperda* cell infected with AcNS1.C37S,C43S in which gold particles are associated with tubules sectioned in transverse (top right) or longitudinal (bottom left) orientation. (b) Section of the cytoplasm of a cell infected with AcNS1.C337S,C340S showing gold-tagged NS1 associated with aggregates of material involving intermediate filaments. No tubules were identified.

FIG. 6. NS1 protein aggregates of materials recovered in the pellet of the gradient-resolved products derived from an AcNT10 infection.

DISCUSSION

Tubules are formed in BTV-infected cells from 4 h postinfection (8). They represent a major constituent of the virus-induced products. However, their function is unknown. Virus-specific tubular structures have been identified for a number of plant viruses such as comoviruses (14), nepoviruses (13), and caulimoviruses (18). It has been postulated that they may be involved in the movement of virus particles through plasmodesmata. BTV tubules are often observed to be closely associated with the host cell cytoskeleton as well as with virions and incomplete particles within cells (3, 11). It has been claimed that NS1 is also associated with extracellular virions, although this is a matter of dispute, since not all researchers have come to the same conclusion. In addition to the lack of knowledge concerning the roles of tubules, it is not known how they are formed, although an involvement of cellular membranes in their polymerization has been suggested (24).

The study presented in this report concerned analyses of mutant NS1 proteins. These were undertaken to identify the requirements for tubule polymerization. Using point mutations of various cysteine residues of NS1, it has been demonstrated that at least some of the cysteines, in particular those corresponding to BTV-10 NS1 amino acids 337 and 340, are essential for tubule formation. These two cysteines are located in a hydrophilic region of the protein, unlike many other conserved cysteine residues that were mutated. Mutant proteins in which these two cysteines were changed to serines gave products that polymerized into ribbon-like structures but did not form tubules. Analysis of single mutants showed that when either C-337 or C-340 was changed to a serine, no tubules were formed. In contrast, two other single mutants (C-104 and C-364) showed no effect on tubule formation, although C-104, like C-337 and C-340, is located in a hydrophilic region of the NS1 protein. These results indicate an important role of C-337 and C-340 in tubule formation. Likewise, the analyses of the amino- and carboxy-terminal deletions of NS1 that have been undertaken also indicate an important role for these regions of NS1 in tubule formation. Which residues of these termini are critical has yet to be determined.

The postulated structure of BTV tubules generated by using cryoelectron microscopy and image processing techniques (7) has revealed that tubules are composed of helically coiled ribbons of NS1 involving dimeric structures. Also, it has been observed that under certain conditions, tubules unwind, forming ribbon-like extensions. These ribbon-like extensions resemble those observed for the deletion mutants and the C-337 and C-340 mutants reported in this paper. As shown by studies involving analyses of thin sections of infected cells, these ribbon-like structures are not due to unwinding or dissociation of preformed tubules during cell homogenization and subsequent purification of NS1 protein. Hewat et al. (7) proposed a model for the winding-unwinding process in which the bonding between the filaments in the direction of the tubule axis was weaker than the bonding within the filaments. The data obtained from our studies support this model if the C-337 and C-340 residues as well as the amino and carboxy termini of NS1 are involved in the formation of tubules from filaments.

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