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Multiple Glycoproteins Synthesized by the Smallest RNA Segment (S10) of Bluetongue Virus

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The genome of bluetongue virus, an orbivirus, consists of 10 double-stranded RNAs, each encoding at least one polypeptide. The smallest RNA segment (S10) encodes two minor nonstructural proteins, NS3 and NS3A, the structures and functions of which are not understood. We have expressed these two proteins in mammalian cells by using the T7 cytoplasmic transient expression system. Using a deletion mutant (lacking the first AUG initiation codon), we have demonstrated that the second initiation codon is used to initiate the synthesis of NS3A protein and that the two initiation codons are responsible for the synthesis not only of NS3 and NS3A but also of high-molecular-weight forms of both proteins. These higher-molecular-weight forms (GNS3 and GNS3A) are glycosylated. We have also demonstrated that the carbohydrate chains of GNS3 and GNS3A could be further modified by heterogeneous extension to polylactosaminoglycan forms. The glycosylated and nonglycosylated forms are found in similar intracellular locations in the Golgi complex. In the presence of cycloheximide, NS3 and NS3A immunofluorescence staining was pronounced in the Golgi complex, confirming that NS3 and NS3A are competent for transport to the Golgi apparatus after synthesis. We conclude that S10 gene products are integral membrane glycoproteins.

Arthropod-borne bluetongue virus (BTV), the etiological agent of bluetongue disease of certain ruminants, replicates in the cytoplasm of a wide range of in vitro cell cultures. BTV is the prototype virus of the genus *Orbivirus* within the family *Reoviridae*. BTV and other orbiviruses (e.g., those causing African horsesickness and epizootic hemorrhagic disease of deer) share many characteristic features with reoviruses and rotaviruses; however, they are distinct in that they multiply in arthropods and vertebrates, causing disease of economic importance in livestock in certain parts of the world. Structurally, these viruses are also different. BTV virions have structural proteins that bear no primary similarity to those of reovirus or rotavirus.

The architecturally complex BTV particle (810 Å [81 nm] in diameter) contains seven structural proteins organized into two protein shells consisting of four consecutive layers (17, 38). The icosahedral, innermost shell encompasses the 10-segment, double-stranded RNA genome and three minor proteins, VP1 (150 kDa), VP4 (76 kDa), and VP6 (36 kDa). This shell consists of 60 copies of one of the two major proteins, VP3 (103 kDa). It forms a scaffold for the outer layer of the viral core (17, 31, 38). This layer is constituted by 780 molecules of the other major core protein, VP7 (39 kDa), organized as 260 trimers on the core surface. The icosahedral core is enclosed by an outer shell containing two other major protein species, VP2 (111 kDa) and VP5 (59 kDa) (40, 46). In addition to encoding the seven structural proteins, the BTV genome encodes four nonstructural proteins, two of which (NS1 and NS2) are readily detectable in BTV-infected cells (18, 19). High-level expression of the 64-kDa NS1 and 41-kDa NS2 and their localization in virus-infected cells have provided some indication of the structure and function and of the location of these proteins (5, 42, 44). In contrast, the two smallest proteins, the 24- to 26-kDa NS3 and NS3A, are difficult to detect in infected cells; hence, little information is available regarding their functions and precise locations.

Both NS3 and NS3A are encoded by BTV segment 10 (S10) and from sequence analyses are estimated to be approximately 25.5 and 24 kDa, respectively (15, 29). To elucidate their structure-function relationships, we have previously expressed NS3 and NS3A of BTV type 10 (BTV-10) in insect cells by using a baculovirus expression system (8). In contrast to the in vitro translation system, in which the two protein products were synthesized in equimolar amounts (35), we have demonstrated that the NS3 protein was the principal product, both in recombinant virus-infected insect cells and in BTV-infected BHK cells, and that the two protein products are closely related to each other (8). Two in-phase initiation codons, one at nucleotide residues 20 to 22 and the other at residues 59 to 61, were assumed to be responsible for initiation of these two protein products, although whether NS3A is indeed the product of the second translation initiation codon or a posttranslational derivative of the NS3 protein was not demonstrated in previous studies.

The predicted sequence of the encoded products of S10 RNA reveals at least two conserved hydrophobic domains (amino acid residues 118 to 147 and 156 to 182, respectively; Fig. 1A), which may serve as membrane-spanning domains. In addition, two potential glycosylation sites (amino acid residues 63 to 65 and 150 to 152, respectively) can also be identified. Recent immunoelectron microscopic studies (20) have revealed that NS3 and NS3A are associated with intracellular smooth-surfaced vesicles and the plasma membrane, suggesting that they may be involved in the final stages of BTV morphogenesis, i.e., the release of BTV from

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infected cells. It has been speculated that NS3 may share some functional similarities with rotavirus glycoprotein NS28, which mediates the budding of rotavirus particles at the rough endoplasmic reticulum (ER) (3, 6, 36). However, whether NS3 and NS3A of BTV are glycosylated like NS28 and whether they indeed have similar functional attributes have not been documented to date.

We therefore embarked on addressing such questions in regard to NS3 and NS3A. To this end, we have expressed these proteins in mammalian cells by using a vaccinia virus-T7 polymerase transient expression system (9). In this study, we have determined the initiation codon for the smaller protein product of the S10 gene, NS3A, and have provided data which clearly demonstrate that the two proteins (NS3 and NS3A) exist in both unmodified and modified forms. The modified forms of NS3 and NS3A are N-linked glycoproteins containing high-mannose sugars. These glycosylated proteins are further modified by the addition of complex heterogeneous carbohydrate chains with characteristics that are similar to those of polylactosaminoglycans. In addition, we have demonstrated the localization of NS3 and NS3A in the ER and Golgi complex of the cytoplasm of infected cells.

MATERIALS AND METHODS

Viruses and cells. U.S. prototype BTV-10 was cloned by plaque formation and propagated in monolayers of BHK-21 cells or BSR cells, using Eagle's minimal essential medium containing 5% (vol/vol) fetal bovine serum. A vaccinia virus recombinant expressing the T7 RNA polymerase was obtained from B. Moss (National Institutes of Health, Bethesda, Md.) and propagated in Vero cells; infectivity titers were measured by plaque assays on confluent monolayers of Vero cells. Vero and HeLa $T4^+$ cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn bovine serum.

DNA manipulations and construction of plasmids. Manipulations of plasmids were carried out as described elsewhere (33). Restriction enzymes, T4 DNA ligase, and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, Ind.). DNA was purified by Geneclean kits (Bio 101 Inc., La Jolla, Calif.). Plasmid pGEM-3Z was purchased from Promega Corp. (Madison, Wis.). To construct the NS3 and NS3A recombinants (designated pGEM-NS3 and pGEM-NS3A in Fig. 1B), pGEM-3Z was digested by *Bam*HI or *Bam*HI and *Hin*dIII and purified; the *Bam*HI-treated fragment was then dephosphorylated. A plasmid (pAcYM1BTV-10.10) containing the cDNA of BTV-10 S10 RNA (8) was digested by *Bam*HI; the inserted DNA was separated in an agarose gel (1%), purified by Geneclean, and then cloned into the *Bam*HI site of the dephosphorylated symmetrical polylinker of pGEM-3Z. The orientation and sequence of the inserted DNA under the control of the T7 promoter of pGEM-3Z were confirmed by sequence analyses. To construct pGEM-NS3A, oligonucleotides were synthesized by the University of Alabama at Birmingham core facility and purified by high-performance liquid chromatography. Two primers were synthesized, an upstream primer (primer 1, 5'-AAGGATCCAAGATGAAA CACAATCAA-3') containing a *Bam*HI site (underlined) followed by the sequence representing nucleotides 56 to 74 of S10 RNA of BTV-10, and a downstream reverse primer (primer 2, 5'-AAAAGCTTTCAGGTTAATGGCATTTC-3') containing the sequence of nucleotides 675 to 709 and a *Hin*dIII site. The primers were used to amplify the NS3A DNA fragment by the polymerase chain reaction (PCR). The amplified DNA fragment which lacked the first ATG but contained the second ATG was digested by *Bam*HI and *Hin*dIII, purified by Geneclean, and then ligated into *Bam*HI- and *Hin*dIII-cut pGEM-3Z downstream of the T7 promoter sequence.

PCR amplification. Selected DNA fragments of S10 of BTV-10 were amplified by PCR with plasmid pGEM-NS3 as the template DNA in a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, Conn.), using the following conditions: primers 1 and 2 each at 30 mM (final concentration), deoxynucleoside triphosphates at 100 μM each, 100 ng of template plasmid DNA, *Taq* DNA polymerase at 2.5 U, and other reaction conditions as specified by the GeneAmp DNA amplification kit instructions in a total volume of 100 μl. The reaction mixtures were overlayed with 75 μl of mineral oil, and reactions were carried out for 24 cycles. Each cycle included a heat denaturation step at 92°C for 1 min, followed by annealing of primers to the DNA template at 50°C for 1 min, and DNA chain extension with *Taq* polymerase at 72°C for 2 min. After the reaction was completed, the PCR products were purified with the Geneclean kits.

T7 polymerase transient expression. HeLa $T4^+$ cells were grown to 85 to 90% confluence and were infected with a recombinant vaccinia virus containing the T7 RNA polymerase gene (VVTF-7) at a multiplicities of infection (MOIs) of 10 to 20 for 1 h at 37°C. The infected cells were washed three times with prewarmed phosphate-buffered saline (PBS), and then 0.5 to 2 ml of serum-free Dulbecco's modified Eagle's medium was added. Recombinant plasmid DNA (5 to 20 μg [0.5 μg/μl of H_2O]) was mixed gently with an equal volume of Lipofectin (Bethesda Research Laboratories, Bethesda, Md.). The mixture was incubated at room temperature for 15 min, added to the infected cells, and incubated for another 8 to 15 h prior to the addition of radiolabel or processing for immunofluorescence analysis (7).

Radiolabeling of cells and immunoprecipitation of viral proteins. Transfected cells were washed twice with PBS and incubated in methionine- and cysteine-free medium for 1 to 2 h. The cells were then labeled with [^{35}S]methionine (100 μCi/ml; NEN, Boston, Mass.) in methionine- and cysteine-free medium for the indicated time periods and chased in Eagle's medium containing 10 mM methionine for the indicated periods. For BTV proteins, Vero cells were infected with BTV-10 at an MOI of 5 for 24 h, and the infected cells were washed with prewarmed PBS twice and labeled with [^{35}S]methionine (50 μCi/ml; NEN) in methionine- and cysteine-free medium for 4 h. For immunoprecipitation, a polyclonal NS3 antibody (8) was preincubated with protein A–Sepharose CL-4B (Promega) at 4°C for 4 h and washed three times with ice-cold cell lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 20 mM EDTA). ^{35}S-labeled infected cells were harvested; nuclei and cell debris were removed by centrifugation at 13,000 rpm for 5 min at 4°C. The supernatant was transferred into a fresh tube and then incubated overnight with the complex of antibody and protein A at 4°C. The precipitates were pelleted, washed three times with cold cell lysis buffer, and resuspended in Laemmli sample buffer (28) with or without β-mercaptoethanol for SDS-polyacrylamide gel electrophoresis (PAGE).

Tunicamycin treatment of infected cells. Tunicamycin (2.5 μg/ml) was added to infected cells, and incubations continued for 4 to 6 h prior to the addition of [^{35}S]methionine. Cells were harvested and processed as described above.

Endoglycosidase treatment. Immunoprecipitated samples

were resuspended in 200 μl of incubation buffer (0.1 M sodium acetate [pH 5.5] for endoglycosidase H [endo H] or 50 mM potassium phosphate [pH 7.0]–30 mM EDTA–1% Triton X-100–0.2% SDS–1% 2-mercaptoethanol) and then divided into two equal parts. Samples were incubated with or without 10 mU of endo H (Miles Laboratories, Inc.) or 100 mU of endo F (Boehringer Mannheim) per sample for 14 to 16 h at 37°C and then centrifuged at 15,000 × *g* for 5 min. The precipitates were resuspended in Laemmli sample buffer, boiled for 5 min, and then analyzed by SDS-PAGE under reducing conditions.

Western immunoblot analysis of BTV-infected cells. To prepare BTV proteins, Vero cells were infected with BTV-10 at an MOI of 5 for 48 h, and the infected cells were harvested, washed with PBS, and collected by centrifugation. The proteins were separated by SDS-PAGE, electroblotted onto an Immobilon membrane, and incubated with NS3 antibodies as described previously (8). Immunoreactive bands were visualized by using an alkaline phosphatase conjugate as described previously (8).

SDS-PAGE and isolation of protein bands. SDS-PAGE was performed with 10 or 15% polyacrylamide slab gels (0.75 mm thick) with an acrylamide/bisacrylamide ratio of 30:1 as previously described (28). Labeled polypeptides were detected by autoradiography of dried gels (Kodak X-Omat XAR-5 film) at −70°C.

For recovery of the immunoprecipitated bands from SDS-PAGE, the bands were excised from the gel and minced finely in a microcentrifuge tube, and 400 μl of gel elution buffer (50 mM ammonium bicarbonate [pH 7.8], 0.1% SDS) was added. Samples were incubated at 37°C for 2 h, the acrylamide fragments were pelleted by centrifugation and washed with 200 μl of the gel elution buffer, and then the two supernatants were pooled. The eluted proteins were concentrated by drying in a Speed-Vac vacuum concentrator.

Indirect immunofluorescence. Vero cells grown on glass coverslips were either infected with BTV-10 at an MOI of 5 and incubated for 24 h or infected with VVTF-7 at an MOI of 10 for 30 h and then transfected with pGEM-NS3/NS3A for 6 h at 37°C. Alternatively, tunicamycin (2.5 μg/ml) was added to the BTV-infected cells 18 h postinfection, and incubation continued for another 6 h. Similarly, tunicamycin and/or cycloheximide (50 μg/ml) was added to VVTF-7-infected and pGEM-NS3/NS3A-transfected cells after 4 h of transfection, and incubation continued for another 4 h prior to fixation. Cells were washed with warm PBS and fixed with cold ethanol containing 5% acetic acid for 3 min at 20°C. Fixed cells were washed with PBS, then reacted with the anti-NS3 mouse serum or anti-BTV-10 virion rabbit serum (lacking NS3 antibody) at 37°C for 1 h, and incubated at 37°C for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (NS3) or goat anti-rabbit immunoglobulin G (BTV). After a final washing, coverslips were mounted and observed with a Nikon Optiphot microscope equipped with a modified B2 tube.

RESULTS

Expression of the NS3 and NS3A proteins in the T7 transient expression system. Since BTV-infected mammalian cells produced a very low level of the NS3 and NS3A proteins, an alternate mammalian expression system was adopted for expression of these proteins. This system is based on infection of the cells with a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase (VVTF-7) and subsequent transfection with plasmid DNA containing the gene to be expressed under the control of a T7 promoter (9). Transcription of the gene is mediated by the T7 polymerase produced in the cytoplasm by the recombinant vaccinia virus (9). To use the T7 expression system, a transient expression plasmid, pGEM-NS3, was constructed (Fig. 1B). HeLa $T4^+$ cells were infected with VVTF-7 and transfected with the recombinant pGEM-NS3. To determine whether BTV-derived proteins were synthesized, cells were radiolabeled at 15 h posttransfection and proteins were immunoprecipitated with polyclonal NS3 antibody. When the precipitated samples were analyzed by SDS-PAGE, a strong band equivalent to the 26-kDa NS3 and a faint band equivalent to the 24-kDa NS3A were identified as expected (8). In addition, another strong band, of higher apparent molecular size than NS3 (ca. 30 kDa), with an underlying second faint band (ca. 29 kDa), was detected (Fig. 2, lane 1). To determine whether the 29- to 30-kDa bands were generated by intermolecular interactions (e.g., via disulfide linkage) of NS3 and NS3A, a sample was analyzed in the absence of reducing agent. The migration patterns of all of the bands were similar both in the presence and absence of reducing agent (Fig. 2, lane 2), indicating that the 29- to 30-kDa bands are higher-molecular-weight forms of NS3 or NS3A.

Biogenesis of the NS3A protein. Since the experiments described above demonstrate that NS3A is expressed from the full-length S10 and recognized by NS3 antibody protein, it was of interest to determine the mechanism of biogenesis of NS3A. Therefore, a second recombinant plasmid, pGEM-NS3A, lacking the first ATG but containing the second in-frame translation initiation codon, was constructed (Fig. 1) and similarly expressed in the T7 system. When the pGEM-NS3A-transfected cells were analyzed as described above, a strong band of the 24-kDa NS3A was expressed (Fig. 2; compare lanes 1 and 3). In addition, a 29-kDa product similar in position to the faint band seen in the NS3 construct was produced. As before, the mobilities of these bands did not differ in the absence of reducing agent (Fig. 2, lanes 3 and 4). These results demonstrate that the second initiation codon can be used to initiate NS3A, that a higher-molecular-weight form of NS3A is also produced, and that in the unmodified construct, the NS3 forms predominate over the NS3A species.

The NS3 and NS3A proteins are N-linked glycoproteins. Since both pGEM-NS3 and pGEM-NS3A genes produced products larger than the predicted 26- to 24-kDa NS3 and NS3A protein products, and since there are two possible N-linked glycosylation sites in S10 (Fig. 1A) (20, 29), it was of interest to determine whether these larger polypeptides were due to N-linked glycosylation. VVTF-7-infected HeLa $T4^+$ cells were transfected with the two plasmids, labeled with [^{35}S]methionine, and immunoprecipitated with anti-NS3 antibody, and an aliquot of each protein product was digested by endo F, which can remove all forms of N-linked carbohydrate chains. The digested products were then analyzed by SDS-PAGE. In the treated profiles (Fig. 3A, lanes 2 and 4), the 29- to 30-kDa protein products of NS3 and NS3A disappeared completely and the amounts of the 26-kDa NS3 and 24-kDa NS3A proteins increased significantly, indicating that these products were generated from digestion of the higher-molecular-weight bands. The results clearly demonstrated that the high-molecular-weight bands are the glycosylated forms of the NS3 and NS3A polypeptides.

To confirm these results by another approach, we treated the transfected cells with various amounts of tunicamycin prior to radiolabeling. The protein products were analyzed similarly. The data for tunicamycin treatment were similar to

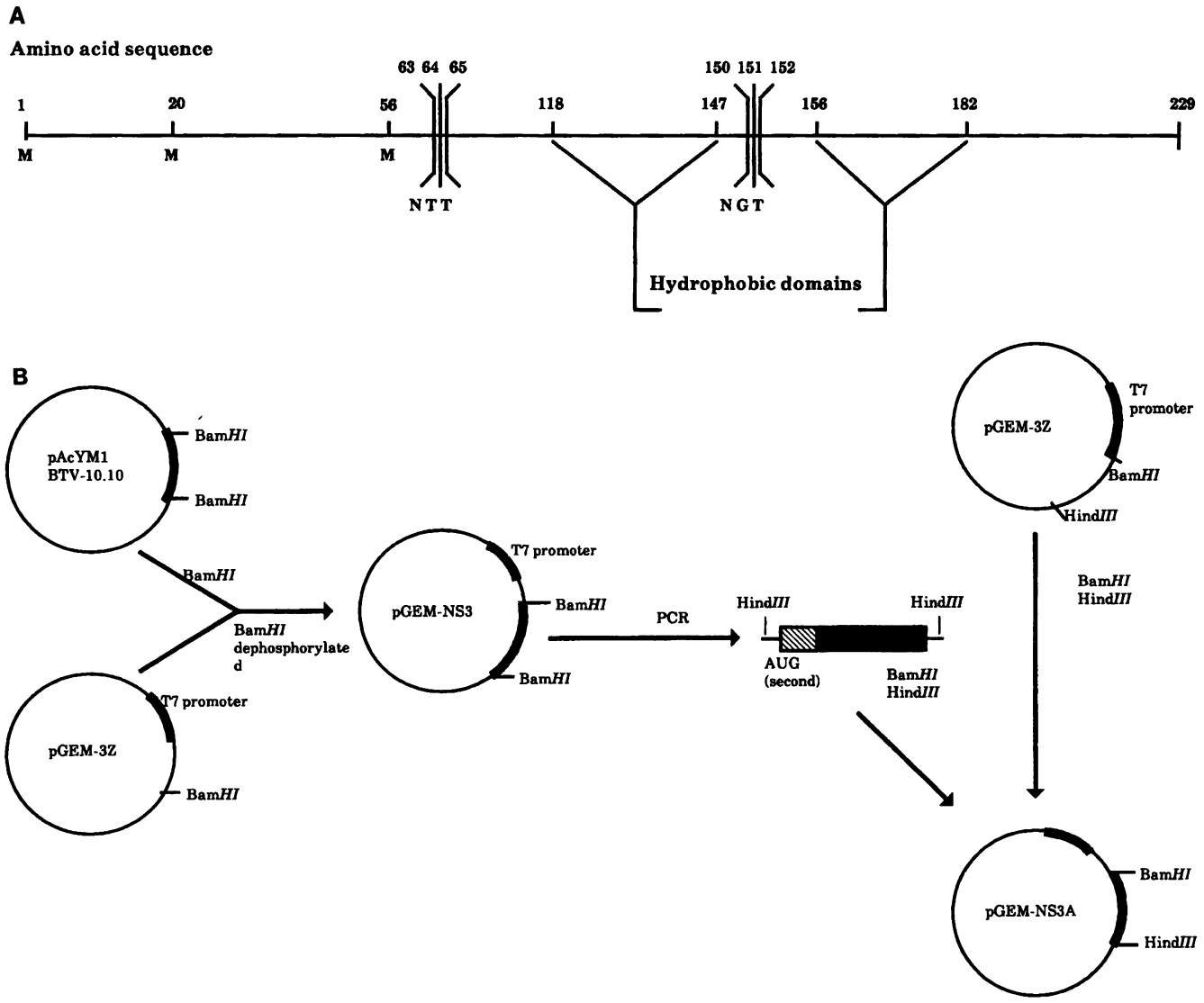

FIG. 1. (A) Predicted amino acid sequence of S10 gene products. The locations of the two hydrophobic domains and of potential N-linked glycosylation sites at positions 63 to 65 and 150 to 152 are shown. (B) Construction of plasmids pGEM-NS3 and pGEM-NS3A.

those for endo F digestion; i.e., the higher-molecular-weight products of NS3 (Fig. 3B) and NS3A (Fig. 3C) disappeared completely, with large accumulations of the nonglycosylated forms of the proteins. In view of these results, we have designated the glycosylated forms of the two proteins GNS3 and GNS3A, respectively (Fig. 2 and 3). Similar glycosylated and nonglycosylated forms of NS3 and NS3A were also identified in BTV-infected cells (data not shown).

NS3 and NS3A are modified with polylactosaminoglycan-like complex carbohydrate chains. To determine whether glycosylated forms of NS3 and NS3A are as stable as the nonglycosylated products, we performed pulse-chase experiments. The transfected cells were labeled with [^{35}S]methionine for 1 h and then chased in minimal essential medium containing 10 mM methionine. The products were analyzed by SDS-PAGE following immunoprecipitation. Much to our surprise, we observed that the glycosylated forms were not as stable as the unmodified proteins in the transfected cells. Instead, heterogeneous diffuse bands in the molecular size

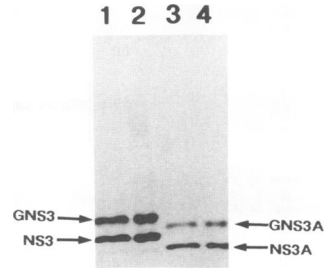

FIG. 2. Expression of NS3 and NS3A. HeLa T4$^+$ cells were infected with VVTF-7 at an MOI of 20, then transfected with 20 μg of pGEM-NS3 (lanes 1 and 2) or pGEM-NS3A (lanes 3 and 4) plasmid DNA in Lipofectin, and incubated for 14 h. Infected cells were then labeled with [^{35}S]methionine (100 μCi/ml) for 30 min at 37°C. The cells were lysed, and the NS3 (lanes 1 and 2) and NS3A (lanes 3 and 4) proteins were immunoprecipitated from the cell lysate with anti-NS3 mouse serum and analyzed by SDS-PAGE (10% polyacrylamide gel) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of β-mercaptoethanol. For positions of the proteins relative to molecular weight markers, see Fig. 4 and 5.

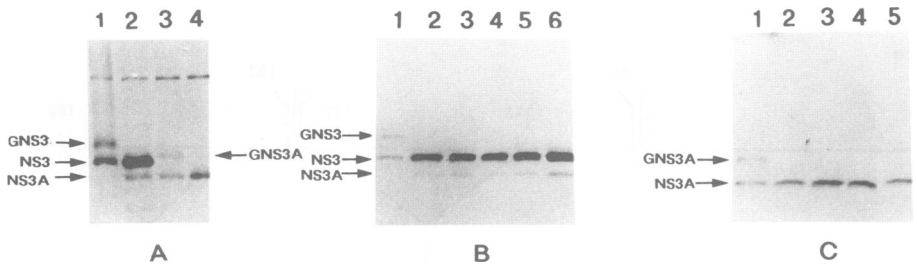

FIG. 3. Glycosylation of NS3 and NS3A proteins expressed by transient expression systems. Vaccinia virus-infected HeLa T4$^+$ cells were transfected with plasmid pGEM-NS3 or pGEM-NS3A as described in the text. (A) At 14 h after transfection, cells were labeled with [^{35}S]methionine (100 μCi/ml) for 1 h at 37°C. The cells were lysed and immunoprecipitated with anti-NS3 mouse serum. The precipitated samples were divided into two parts; one was treated with 100 mU of endo F, the other was used as a control, and both were incubated at 37°C for 14 h. The samples were then analyzed by SDS-PAGE (15% polyacrylamide gel) under reducing conditions. Lanes: 1, control NS3; 2, endo F-treated NS3; 3, control NS3A; 4, endo F-treated NS3A. At 12 h after transfection with pGEM-NS3 (B) or pGEM-NS3A (C), cells were treated with tunicamycin (lanes 2 to 6) for 4 h and labeled with [^{35}S]methionine (100 μCi/ml) for 1 h at 37°C, and proteins were immunoprecipitated. Lanes: 1, control; lanes 2 to 5, treated with tunicamycin at 1.25 μg (lane 2), 2.5 μg (lane 3), 5 μg (lane 4), or 10 μg (lane 5). For positions of the proteins relative to molecular weight markers, see Fig. 4 and 5.

range of ca. 32 to 100 kDa appeared, which increased in amount with the time of chase, while the GNS3 and GNS3A protein bands concomitantly decreased (Fig. 4). The results suggested that the carbohydrate chain(s) could be further modified by heterogeneous extension (12, 13, 21, 47, 48). To examine whether there is a relationship between the heterogeneous diffuse bands and the NS3 and NS3A proteins, HeLa T4$^+$ cells transfected with pGEM-NS3 were labeled for 4 h and the proteins were immunoprecipitated with anti-NS3 antibody. The protein bands were then separated on SDS–10% polyacrylamide gels and isolated in two groups; one group included the bands of 18 to 32 kDa (NS3, GNS3, NS3A, and GNS3A), and a second group contained bands of 32 to 100 kDa (i.e., the putative further modified forms of the proteins). Each sample was divided into three aliquots; one was treated with endo F, one was treated with endo H, and the third was kept as a control. When analyzed by SDS-PAGE, GNS3 and GNS3A disappeared upon endo F digestion, as expected for the removal of N-linked carbohydrates and deglycosylation of the proteins. Concomitantly, two strong lower-molecular-weight bands with the same

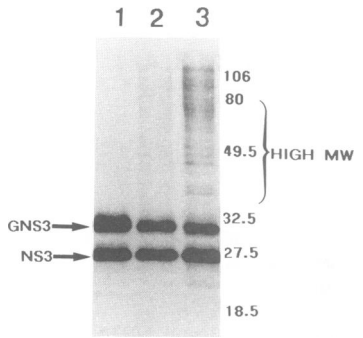

FIG. 4. Modification of glycoproteins to heterogeneous higher-molecular-weight (HIGH MW) forms. HeLa T4$^+$ cells were transfected with plasmid pGEM-NS3 and after 16 h were labeled with [^{35}S]methionine (100 μCi/ml) for 60 min at 37°C. The cells were chased for 0 (lane 1), 60 (lane 2), or 120 (lane 3) min in the presence of excess methionine, and proteins were immunoprecipitated with anti-NS3 mouse serum at 37°C. The samples were analyzed by SDS-PAGE (15% polyacrylamide gel) under reducing conditions. The positions of molecular weight markers (in kilodaltons) run alongside are indicated.

molecular weights as the unglycosylated NS3 and NS3A proteins appeared (Fig. 5A, lane 4). However, the results obtained from endo H treatment of the high-molecular-weight bands were very different. Most of them, unlike the GNS3 and GNS3A proteins, were resistant to digestion by endo H (Fig. 5A, lane 5). In contrast, endo F treatment resulted in their conversion to lower-molecular-weight bands similar to unglycosylated NS3 and NS3A (Fig. 5A, lane 2). To investigate whether similar high-molecular-weight bands could be detected from BTV-derived NS3 protein products, protein samples from BTV-infected cells were separated by SDS-PAGE (10% polyacrylamide gel) and analyzed by Western immunoblotting with anti-NS3 antiserum. As shown in Fig. 5B, in addition to NS3 and NS3A, a number of slower-migrating bands reacted with the anti-NS3 antiserum. However, as expected, these bands disappeared upon endo F treatment.

The results suggest that the heterogeneous protein forms arise through further maturation of carbohydrates from high-mannose to polylactosaminoglycan forms, which are probably processed in the Golgi apparatus (48).

Localization of NS3 and NS3A proteins in infected cells. The results obtained from the experiments described above demonstrate that S10 gene products of BTV are modified by N-linked glycosylation, indicating that these products are synthesized by membrane-attached ribosomes. In view of this finding, it is highly likely that the proteins are integral membrane glycoproteins. Since the cytoplasmic compartments for membrane-bound proteins are different from those of the unbound proteins, we investigated the location of the proteins in transfected cells by immunofluorescence and by comparison with BTV-infected cells. In contrast to nontransfected cells (Fig. 6A), NS3 and NS3A were observed in the ER of transfected cells (Fig. 6B), with indications that the proteins were also present in the Golgi apparatus (Fig. 6C), suggesting that NS3 and NS3A can apparently achieve a transport-competent conformation in the ER and progression to components of the Golgi apparatus. Similar ER and Golgi locations of NS3 and NS3A were also found in BTV-infected cells (Fig. 7D to F). When cycloheximide was added 4 h posttransfection and cells were incubated for a further 4 h, the NS3 and NS3A staining showed a more pronounced Golgi apparatus-like staining, confirming that the NS3 and NS3A products are competent for transport to the Golgi apparatus after synthesis (Fig. 7C). To examine

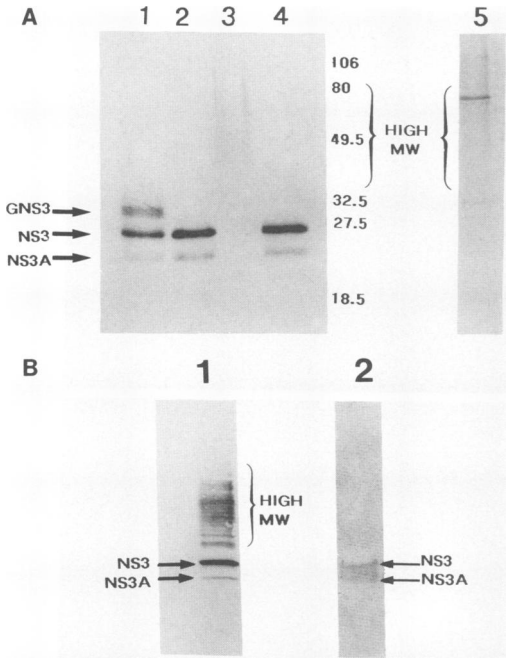

FIG. 5. Endoglycosidase treatment of the polylactosaminoglycan-modified proteins. (A) At 16 h after transfection with pGEM-NS3, HeLa T4$^+$ cells were labeled with [^{35}S]methionine for 3 h at 37°C, lysed, and immunoprecipitated with mouse anti-NS3 serum. The specific proteins were separated by SDS-PAGE (10% polyacrylamide gel) under reducing conditions and recovered from the gels. One group (sample 1) corresponded to 18 to 32 kDa; another (sample 2) corresponded to 32 to 100 kDa (HIGH MW). Each group was divided into three aliquots; one was treated with 100 mU of endo F (lanes 2 [sample 1]) and 4 [sample 2]), another was treated with 10 mU of endo H (lane 5 [sample 2]), and the third served as a control (lanes 1 [sample 1] and 3 [sample 2]). The samples were incubated for 16 h at 37°C and then analyzed by SDS-PAGE (15% polyacrylamide gel) under reducing conditions. (B) Vero cells were infected with BTV-10 at an MOI of 5 PFU per cell, and cell lysates were processed as described in the text. Proteins were separated by SDS-PAGE (10% polyacrylamide gel) (lane 1), electroblotted onto an Immobilon membrane, and reacted with anti-NS3 antibody. Bound antibody was detected by using an alkaline phosphatase conjugate (lane 2). The positions of molecular weight markers (in kilodaltons) run alongside are indicated in panel A.

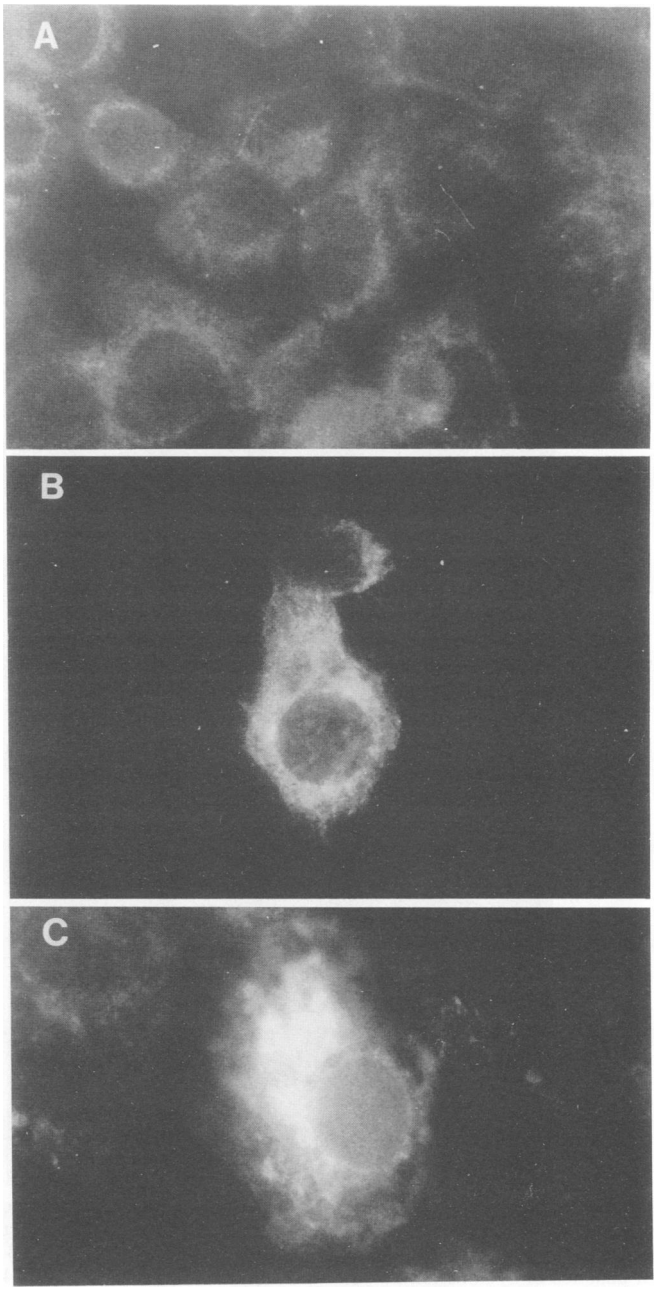

FIG. 6. Intracellular localization of the expressed NS3 and NS3A proteins in transfected cells. Vero cells grown on coverslips were infected with VVTF-7 for 30 h at 37°C and transfected with pGEM-NS3 or pGEM-NS3A, incubation continued for 4 h, and the cells were fixed and then reacted with NS3 antibody followed by fluorescein-labeled second antibodies. (A) VVTF-7-infected control cells; (B) similar cells transfected with pGEM-NS3, showing the presence of NS3 and NS3A in the ER (similar data were obtained with pGEM-NS3A); (C) cells transfected with pGEM-NS3A, showing the presence of NS3A in the Golgi complex (similar data were obtained with pGEM-NS3).

whether the addition of carbohydrate chains to the NS3 proteins was necessary for them to be transported intracellularly, infection was performed in the presence of tunicamycin, and the protein products were detected by indirect immunofluorescence staining (Fig. 7). The distribution of NS3 and NS3A patterns both in transfected cells and in BTV-infected cells (Fig. 7C and D) showed no detectable difference in the presence or absence of tunicamycin. The staining patterns of NS3A were similar to those of NS3 and NS3A in the transfected cells, suggesting that their localizations were similar (Fig. 7). The results indicate that glycosylated and nonglycosylated forms of NS3 and NS3A have similar intracellular locations in the ER and components of the Golgi apparatus.

DISCUSSION

The genome of BTV consists of 10 double-stranded RNA segments, each of which except the smallest (S10) encodes one protein product. Previously we have expressed the S10 gene by using a recombinant baculovirus and have shown that it encodes at least two closely related proteins, NS3 and NS3A, with almost identical peptide maps. In this study, we have used the vaccinia virus-based T7 transient expression

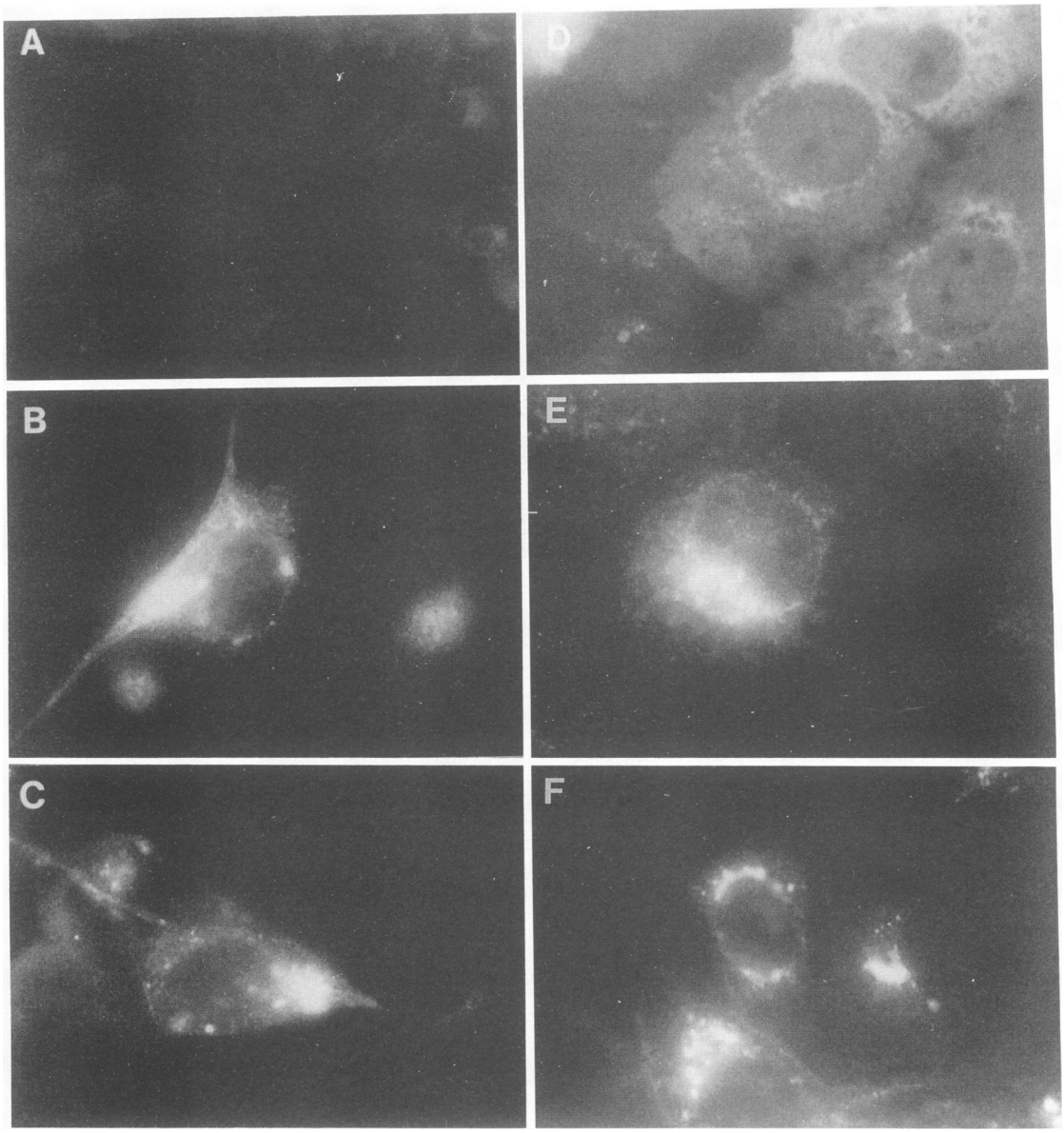

FIG. 7. Effects of cycloheximide and tunicamycin on the intracellular localization of NS3 and NS3A proteins. BTV-infected Vero cells were treated 18 h postinfection with tunicamycin (2.5 μg/ml) and incubated for a further 6 h before analysis. Cells were fixed and stained with antibody. VVTF-7-infected cells were transfected with pGEM-NS3 (or pGEM-NS3A); cells were treated with tunicamycin in the presence or absence of cycloheximide at 4 h posttransfection and examined 4 h later. (A) VVTF-7-infected control cells treated with anti-NS3/NS3A antibody; (B) NS3 and NS3A in cells transfected with pGEM-NS3 (pGEM-NS3A gave similar results) treated with anti-NS3/NS3A antibody; (C) NS3 and NS3A in pGEM-NS3-transfected cells incubated in the presence of cycloheximide (tunicamycin and pGEM-NS3A transfections gave similar results); (D) BTV-infected cells treated with anti-BTV antiserum (i.e., lacking antibodies to NS3 and NS3A and showing a more general cytoplasmic distribution of viral gene products than that observed with anti-NS3/NS3A antibody); (E) BTV-infected cells treated with anti-NS3/NS3A antibody; (F) BTV-infected cells incubated in the presence of tunicamycin (cycloheximide gave similar results) and later treated with anti-NS3/NS3A antibody.

system to determine the biogenesis of NS3 and NS3A and to analyze their structural characteristics further in order to understand their possible functions in virion morphogenesis.

Our studies have demonstrated that NS3A is not simply a cleavage product of NS3; it can be initiated independently from the second ATG codon of the S10 gene. According to the recent hypothesis of Kozak (22–26), protein translation in higher eukaryotes is modulated at the level of initiation by five aspects of mRNA structure: (i) the m^7G cap, (ii) the primary sequence or context surrounding the AUG codon, (iii) the position of the AUG codon, (iv) secondary structure, both upstream and downstream from the AUG codon, and (v) leader length. It is postulated that GCCA/GCCAUGG is the optimal context for initiation of translation in higher eukaryotes. Purines (preferable an A in position −3 and a G in position +4) have the strongest effects, modulating translation at least 10-fold. We analyzed the context surrounding the first and second AUGs from the known S10 sequences

TABLE 1. Contexts surrounding the first and second AUGs of different orbiviruses

Virus	First AUG codon		Second AUG codon	
	Position	Sequence[a]	Position	Sequence
BTV-10 (United States)	20–22	GCCAUGC	59–61	AAGAUGA
BTV-1				
South Africa	20–22	GCCAUGC	59–61	AAAAUGA
Australia	20–22	GCCAUGC	59–61	AAAAUGA
African horse sickness virus				
Type 3	20–22	GUCAUGA	53–55	AUGAUGC
Type 9	19–21	GUCAUGA	52–54	AGUAUGC
Palyam virus	19–21	GACAUGU	52–54	GCGAUGA
Broadhaven virus	18–20	ACAAUGC	36–38	GAGAUGA

[a] Sequences representing putative initiation codons are underlined, and nucleotides at positions −3 and +4 relative to the AUG are in bold type.

(15, 29, 37, 45) of different orbiviruses (Table 1). These data indicated that both the first and second AUG initiation codons reside in weak contexts, which may be responsible for the reduced efficiency of the translation of NS3, resulting in a low level of synthesis of the NS3A protein. When two proteins are encoded from overlapping open reading frames of a single mRNA, the first AUG codon is generally not in an optimal context. In such bifunctional mRNAs, the first AUG codon deviates from the consensus sequence in either or both the −3 and +4 positions. Leaky scanning may result from such suboptimal contexts and thereby enable some viral mRNAs to produce two proteins by initiating at both initiation codons (22, 25, 49). It is likely that S10 serves as a bifunctional mRNA, perhaps as a result of the weak context that surrounds the first AUG codon. However, the data that we have obtained indicate that the first AUG is the preferred codon, since little NS3A is observed and in the absence of the first initiation codon, the level of NS3A expression increased significantly.

We have demonstrated that major portions of both NS3 and NS3A are modified to yield N-linked glycoproteins containing endo H-sensitive high-mannose-type sugars (41). Both proteins are further converted to endo H-resistant higher-molecular-weight products. The complex carbohydrate chain modification of the higher-molecular-weight forms of NS3 and NS3A appears to have the properties of polylactosaminoglycan-modified proteins (12, 43, 48). Both low- and high-molecular-weight glycosylated forms of NS3 and NS3A have also been identified in BTV-infected cells. Although NS3 gene products were expressed previously by both recombinant baculoviruses (8) and recombinant yeast cells (34), glycosylated forms of NS3 gene products were not detected, suggesting that glycosylation of proteins in insect and yeast cells may not be as efficient as in mammalian cells (27, 30).

Data presented here indicate that NS3 and NS3A are probably integral membrane proteins (11, 12, 14, 39) and that the carbohydrate chains on NS3 and NS3A may be similar to those found on band 3, the anion-exchange protein of the erythrocyte membrane (4, 13), or on influenza B virus NB integral membrane glycoprotein (48). Like NS3 and NS3A, the NB protein is modified by endo F-sensitive but endo H-resistant, N-linked polylactosaminoglycan chains that are attached to a mannose core oligosaccharide (reviewed in references 10 and 48). However, further studies are needed to confirm this view.

It was observed that the intracellular localization of the NS3 gene products was different from that of the viral structural proteins. In BTV-infected cells, the typical ER- and Golgi apparatus-like pattern of labeling was detected by anti-NS3 antibody (8). When the NS3 and NS3A proteins were produced independently of other BTV proteins in the T7 transient expression system, their location in transfected cells was similar to that in BTV-infected cells. The data indicate that maturation of NS3 and NS3A takes place in the ER and that mature proteins are competent for transportation to the Golgi apparatus. Data presented here also confirmed that the S10 gene products exist in both modified and unmodified forms. It has been suggested that N-linked glycosylation might play a role in conferring transport competence to some integral membrane proteins (16, 32) and that this posttranslational modification might in some instance provide a signal for transport out of the ER. However, addition of tunicamycin or cycloheximide did not prevent the transportation of NS3 and NS3A proteins to the Golgi complex. Therefore, N-linked glycosylation of the NS3 gene products is not required for their intracellular transport.

Our results indicate that the N-linked glycoproteins attached to the high-mannose oligosaccharides are further modified into polylactosaminoglycan proteins with the heterogeneous carbohydrate chains. It has been reported that cellular glycoproteins with such polylactosaminoglycans are localized in the plasma membrane (10) and lysosomal membranes (11). Influenza B virus NB has also been shown to be a polylactosaminoglycan membrane protein (47). It is therefore highly likely that the NS3 gene products are transported to the plasma membrane, although more data are needed to confirm this possibility. Nevertheless, it is clear that NS3 and NS3A are highly glycosylated, although how this feature relates to their role in virus maturation is not known. It is noteworthy in this context that S10 RNA of rotavirus (SA11) also encodes a glycosylated nonstructural protein with a molecular size of 20 kDa (6). The mature glycoprotein not only is an integral membrane protein of the ER but also contains two sites where N-linked high-mannose oligosaccharides are added (1, 2). However, NS28 contains a noncleavable signal sequence, and the amino terminus of the molecule is maintained in the membrane whereas the carboxyl terminus extends into the cytoplasm of infected cells. The cytoplasmic domain of NS28 has been suggested to be involved in the morphogenesis of virus particles that mature by budding through the ER membrane. Glycosylation of NS28 has been shown to be required for removal of the transient envelope from budding particles. It will be interesting to determine whether NS3 is involved in similar processes in BTV morphogenesis.

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