VIROLOGY 209, 624–632 (1995)

The Multimeric Nonstructural NS2 Proteins of Bluetongue Virus, African Horsesickness Virus, and Epizootic Hemorrhagic Disease Virus Differ in Their Single-Stranded RNA-Binding Ability

J. M. UITENWEERDE,* J. THERON,† M. A. STOLTZ,* and H. HUISMANS*,[1]

*Department of Genetics and †Department of Microbiology and Plant Pathology, University of Pretoria, 0002, South Africa

Received September 19, 1994; accepted April 4, 1995

The structure and single-stranded (ss) RNA-binding by the nonstructural protein NS2 of three different orbiviruses were studied and compared. African horsesickness virus (AHSV), bluetongue virus (BTV), and epizootic hemorrhagic disease virus (EHDV) were analyzed in recombinant baculovirus-infected cells and in cells infected with BTV and AHSV. Sedimentation analysis and nonreducing SDS–PAGE revealed that NS2 of all three orbiviruses is a 7S multimer with both inter- and intramolecular disulfide bonds, probably consisting of six or more NS2 molecules. The 7S NS2 multimer of all three viruses binds ssRNA but there is a marked disparity in the ssRNA-binding ability between the three proteins. At physiological salt concentration, BTV NS2 binds ssRNA very efficiently, whereas AHSV NS2 shows only a low efficiency for binding ssRNA. EHDV NS2 binds with intermediate efficiency. The result was the same irrespective of whether poly(U)–Sepharose or viral mRNA was used, indicating that ssRNA-binding by NS2 is nonspecific. The difference in RNA-binding ability may be related to the α-helix content of the respective proteins. NS2 of BTV has the highest predicted α-helix content followed by EHDV and AHSV. The ability of the NS2 proteins to form virus inclusion body-like structures in baculovirus-infected cells is not affected by the ssRNA-binding disparity.

INTRODUCTION

Bluetongue virus (BTV), African horsesickness virus (AHSV), and epizootic hemorrhagic disease virus (EHDV) are members of the orbivirus genus in the *Reoviridae* family. Each virus has a genome consisting of 10 segments of double-stranded (ds) RNA. BTV, the prototype virus, has been studied extensively, but most of the early events in viral replication are still not well characterized. After the virus has entered the cell, it is converted to a core particle (Huismans *et al.*, 1987b), which transcribes the dsRNA segments into mRNAs (Van Dijk and Huismans, 1988). The mRNAs either are translated or are used as templates for synthesis of dsRNA progeny genomes. Since dsRNA is only associated with viral particles (Brookes *et al.*, 1993), selection and encapsidation of progeny genomes probably occur at the single-stranded (ss) RNA level. Single-stranded RNA-binding proteins are therefore likely to play an important role in virus morphogenesis. The mechanism whereby the 10 different mRNAs are selected and encapsidated is, however, not known.

The sites of virus synthesis in BTV- and AHSV-infected cells are virus inclusion bodies (VIBs) (Lecatsas, 1968; Brookes *et al.*, 1993). In the case of BTV, these VIBs have been shown to consist mainly of the nonstructural protein NS2 (Eaton *et al.*, 1987). BTV NS2 is furthermore known to bind ssRNA (Huismans *et al.*, 1987a; Thomas *et al.*, 1990) and is thought to be subsequently involved in the selection and condensation of the mRNA segments into precursor viral particles.

The NS2-encoding genes of BTV, AHSV, and EHDV have been cloned and sequenced (Hall *et al.*, 1989, Van Staden *et al.*, 1991). Two other members of the *Reoviridae* family also have nonstructural proteins with ssRNA-binding ability, namely σNS of reovirus (Huismans and Joklik, 1976) and NSP3 (NS34) of rotavirus (Boyle and Holmes, 1986). The cognate ssRNA-binding proteins of these different genera do not show sequence similarity, nor do they have any recognized ssRNA-binding motifs. However, they do share a short consensus motif (Van Staden *et al.*, 1991). Both NSP3 and σNS have been shown to multimerize (Mattion *et al.*, 1992; Gomatos *et al.*, 1980) and to bind the 3′ ends of viral mRNA (Poncet *et al.*, 1993; Stamatos and Gomatos, 1982). In this study we have examined the structure and ssRNA-binding of the NS2 proteins of three different orbiviruses, AHSV, EHDV, and BTV, by characterization of the baculovirus-expressed NS2 proteins, as well as the NS2 proteins from cells infected with either BTV or AHSV. Evidence is presented for the first time that NS2 multimerizes by means of intermolecular and intramolecular disulfide bonds in the form of a protein with a sedimentation value of approximately 7S in both the orbivirus and baculovirus expression systems. This 7S NS2 multimer binds ssRNA *in vitro*. However, significant differences were found in the ability of BTV, EHDV, and AHSV NS2 to bind ssRNA, irrespective of the origin of the template — viral or synthetic. This difference in RNA-binding did not affect the ability of the proteins to accu-

[1] To whom reprint requests should be addressed.

0042-6822/95 $6.00

mulate into VIB-like structures when expressed in baculovirus-infected cells.

METHODS

Cells and viruses

Wild-type baculovirus (AcNPV) and recombinant baculoviruses were grown and analyzed in confluent monolayers and spinner cultures of *Spodoptera frugiperda* (Sf9) cells at 27° in Grace's medium containing 10% (v/v) fetal calf serum (Summers and Smith, 1987). The recombinant baculoviruses AcBTV-10.8, AcAHSV-9.8, and AcEHDV-2.8, which expressed the NS2 proteins of BTV-10, AHSV-9, and EHDV-2, respectively, were constructed as described in Theron *et al.* (1994). BTV-10 and AHSV-9 were assayed either in Vero cells or in BHK-21 and CER cells, respectively, in Eagle's medium with 5% bovine serum at 37°.

Radiolabeling of proteins expressed by recombinant baculoviruses

Sf9 cells were infected with virus at a m.o.i. of 10 PFU/cell and incubated at 28°. At 30–42 hr postinfection, the cells were starved of methionine by incubation for 1 hr in methionine-free Eagle's medium. At the end of the incubation, 30 μCi/ml [^{35}S]methionine (Amersham) in methionine-free medium was added to the cells and the incubation was continued for an additional 2–3 hr.

Preparation of subcellular fractions from infected cells

Subcellular fractions were prepared as described by Huismans *et al.* (1987a). The cells were collected, washed in 1× PBS and resuspended in 0.01 *M* STE–TX buffer (0.01 *M* NaCl, 10 m*M* Tris, pH 7.4, 1 m*M* EDTA, 0.5% Triton X-100) in a concentration of 1×10^8 cells/ml. The nuclei were pelleted at 2000 rpm for 5 min and the supernatant or cytoplasmic fraction (S10) was collected. Soluble (S100) and particulate (P100) fractions were prepared by centrifugation of the S10 through a 1-ml sucrose cushion for 1 hr at 45,000 rpm in a SW50.1 rotor and stored at −70°.

Preparation of polyclonal monospecific serum against NS2

NS2 was purified from preparative SDS–PAGE gels of P100 fractions of recombinant baculovirus-infected insect cells. The gels were stained in 0.05% Coomassie blue G250 in water and destained in water. The NS2 band was excised and macerated and the proteins were eluted in 50 m*M* Tris, pH 8, 1% Triton X, 2% SDS for 1 hr with shaking. The eluate was precipitated with 4 volumes acetone for 1 hr at −70°. The proteins were pelleted by 30-min centrifugation at 18,000 rpm. With each immunization 50 μg of the protein was resuspended in 1× PBS and used to immunize rabbits with ISA-50 adjuvant. The rabbits were immunized three times, spaced 4 weeks apart. The antisera were preabsorbed with AcNPV-infected whole-cell extract and used at 1/1000 dilution.

Electron microscopy

Sf9 cells were infected in spinner cultures with recombinant baculovirus at a m.o.i. of >1 PFU/cell and incubated for 48 hr. Alternatively, monolayers of Vero cells were infected with AHSV-9, incubated for 18 hr at 37°, and harvested with trypsin. The cells from both Sf9 and Vero cultures were pelleted and fixed for 60 min in 5% phosphate-buffered formaldehyde. The pellets were dehydrated in ethanol and embedded in LR White resin. Ultra-thin sections on nickel grids were used for immunogold labeling.

Sections were preabsorbed for 30 min in 1% fat-free milk powder and incubated for 60 min with monospecific anti-AHSV NS2 serum (diluted 1/1000) in 0.1% phosphate-buffered bovine serum albumin (BSA). Preimmune serum was used as negative control. After three washes, a secondary blocking step was performed in 1% phosphate-buffered BSA. The grids were incubated in protein A–gold (14-nm particles) for 45 min, washed, contrasted, and viewed at 60 kV with a Philips EM301.

Indirect immunofluorescent detection of NS2 in infected insect and mammalian cells

Sf9 cells were grown on coverslips, infected with AcBTV-10.8 or AcAHSV-9.8, and incubated for 3 days. Likewise, Vero cells were infected with BTV-10 or AHSV-9 and incubated at 37° for 18 hr. The cells were fixed for 3 min in 5% acetic acid in ethanol. Nonspecific binding of antibody was blocked by incubation in 5% milk powder in 1× PBS for 45 min. The coverslips were covered with 1/1000 dilutions of anti-NS2 serum or naive serum in blocking buffer for 1 hr. The secondary antibody, α-rabbit fluorescein, was added to the samples for 1 hr, and the coverslips mounted in Fluoromount (BDH). The cells were studied by fluorescent microscopy.

Sedimentation analyses

Cytoplasmic fractions, S10 (300 μl), were prepared either from 1×10^7 Sf9 cells infected with one of the three baculovirus NS2 recombinants or from 5×10^7 virus-infected BHK or CER cells. The extracts were layered onto linear 10–40% sucrose gradients in 0.15 *M* STE–TX and centrifuged for 16 hr at 40,000 rpm (SW50.1 rotor). Forty drops per fraction was collected from the bottom of the gradients and analyzed by SDS–PAGE (Laemmli, 1970) or immunoblot. Where indicated, the S10 fractions were treated with 20 μl/ml RNase for 10 min. Alternatively, the NaCl concentration was adjusted to 0.5 *M* NaCl and diluted back to 0.15 *M* NaCl immediately prior to sedimentation analysis.

Immunoprecipitation

[^{35}S]Methionine-labeled S10 fractions (500 μl) were prepared from 2 × 10^6 cells infected with AcBTV-10.8 or AcAHSV-9.8. Either anti-BTV NS2 or anti-AHSV NS2 serum (1 μl) was added to 100 μl of the cell lysate and diluted to 500 μl with 0.01 *M* STE–TX. The samples were shaken for 1 hr at room temperature, after which *Staphylococcus aureus* protein A was added for an additional 1 hr. The protein A with bound immunocomplexes was pelleted, washed three times with 0.01 *M* STE–TX, and finally resuspended either in 1× protein solvent buffer (PSB) (with 50 m*M* β-mercaptoethanol) or in 1× PSB without β-mercaptoethanol and analyzed by SDS–PAGE (Laemmli, 1970) and autoradiography.

Poly(U)–Sepharose binding assay

Cytoplasmic fractions were prepared from 1 × 10^7 Sf9 cells infected with the respective NS2 recombinant baculovirus at a m.o.i. of 10 PFU/cell and labeled with [^{35}S]-methionine at 30–33 hr postinfection. The fractions were loaded on 10–40% sucrose gradients in 0.4 *M* STE–TX and centrifuged as described earlier to obtain unbound NS2. The fractions containing the 7S NS2 multimer were pooled and used to assay RNA-binding.

The assays were performed in the presence of excess amounts of poly(U)–Sepharose 4B (Sigma). A quantity of the appropriate sucrose gradient fraction (≤35 μl) was added to ≤20 mg poly(U)–Sepharose in 500 μl STE–TX, pH 7.5, at NaCl concentrations of between 0.01 and 0.4 *M* as indicated in the text. The proteins were allowed to bind to the poly(U)–Sepharose at room temperature for 30 min with gentle agitation. The poly(U)–Sepharose was collected by centrifugation for 1 min at 2000 rpm and washed twice in the appropriate binding buffer and both the bound and the unbound proteins were quantitated by scintillation counting. The unbound protein was precipitated by acetone as previously described. The precipitates and poly(U)–Sepharose were resuspended in protein solvent buffer and analyzed by SDS–PAGE and autoradiography.

Viral mRNA-binding assay

^{35}S-Labeled NS2 multimers of AcBTV-10.8 and AcAHSV-9.8 were purified as described previously. ^{3}H-Labeled mRNAs of BTV 10 segment 8 and AHSV 6 segment 9 were prepared by standard *in vitro* transcription assays using Bluescribe vectors (Stratagene). Binding assays were performed with excess protein. Samples of 50–100 μl of the NS2-containing fractions were mixed with 1 μg viral mRNA in STE buffer at between 0.05 and 0.35 *M* NaCl. Binding was allowed to occur at 4° for 30 min after which NS2 was immunoprecipitated using monospecific anti-NS2 serum. The amounts of ^{35}S-labeled protein and ^{3}H-labeled RNA in the immunoprecipitate and supernatant were quantitated by scintillation counting.

RESULTS

Sedimentation analysis of NS2

In a preliminary investigation, differences in the sedimentation profiles of the complexes associated with NS2 in BTV- and AHSV-infected cells were observed. This prompted a more detailed investigation in which the sedimentation profiles of baculovirus-expressed NS2 of three different orbiviruses (BTV, AHSV, and EHDV) were compared. Recombinant baculoviruses expressing NS2 of BTV-10, AHSV-9, and EHDV-2 (Alberta) were constructed as previously described (Theron *et al.*, 1994) and were named AcBTV-10.8, AcAHSV-9.8, and AcEHDV-2.8, respectively. Cytoplasmic extracts of recombinant baculovirus-infected cells were prepared in 0.15 *M* STE, centrifuged on sucrose gradients, and analyzed by SDS–PAGE as described under Methods. The results are shown in Fig. 1.

NS2 of AcBTV-10.8 (Fig. 1A) was found to be predominantly associated with very high S value complexes in the pellet. In addition, a heterogenous smear of NS2-containing material was observed in the bottom part of the gradient with the lowest S value in the region of approximately 7S, when compared to the sedimentation of rabbit γ-globulin (6.73S). In the case of NS2 of AcEHDV-2.8 (Fig. 1B), a similar heterogenous distribution of NS2 material was observed, but much less sedimented into the pellet, suggesting that the EHDV NS2 complexes were generally smaller than those of BTV. In the case of NS2 of AcAHSV-9.8 (Fig. 1C), only a small amount of NS2 was found in the pellet and the protein sedimented predominantly with a sedimentation value of approximately 7S (fractions 7 and 8). In each case the position of NS2 was confirmed by immunoblot (result not shown). The difference in sedimentation of the three proteins did not depend either on the amount of NS2 loaded onto the gradient or on the level of expression of NS2 in the baculovirus cells.

To investigate whether the heterogenous sedimentation profiles were due to the formation of either protein–protein or protein–RNA complexes, the baculovirus-infected cell extracts were treated with RNase prior to sedimentation analysis and the result obtained with AcBTV-10.8 is shown in Fig. 2. After treatment, the high S value complexes of BTV NS2 were dissociated and most of the NS2 sedimented as a discrete complex similar to that found in the case of AHSV NS2. Similar results were obtained in the case of EHDV NS2 and also after treatment of both BTV and EHDV NS2 with 0.5 *M* NaCl (result not shown). The sedimentation of AHSV NS2, however, remained largely unchanged after RNase or high-salt treatments (results not shown).

To determine whether these results reflect what is normally observed in BTV- and AHSV-infected cells, the experiments were repeated with NS2 derived from BTV-10- and AHSV-9-infected cell extracts. The sedimentation profiles before and after treatment with either 0.5 *M* NaCl

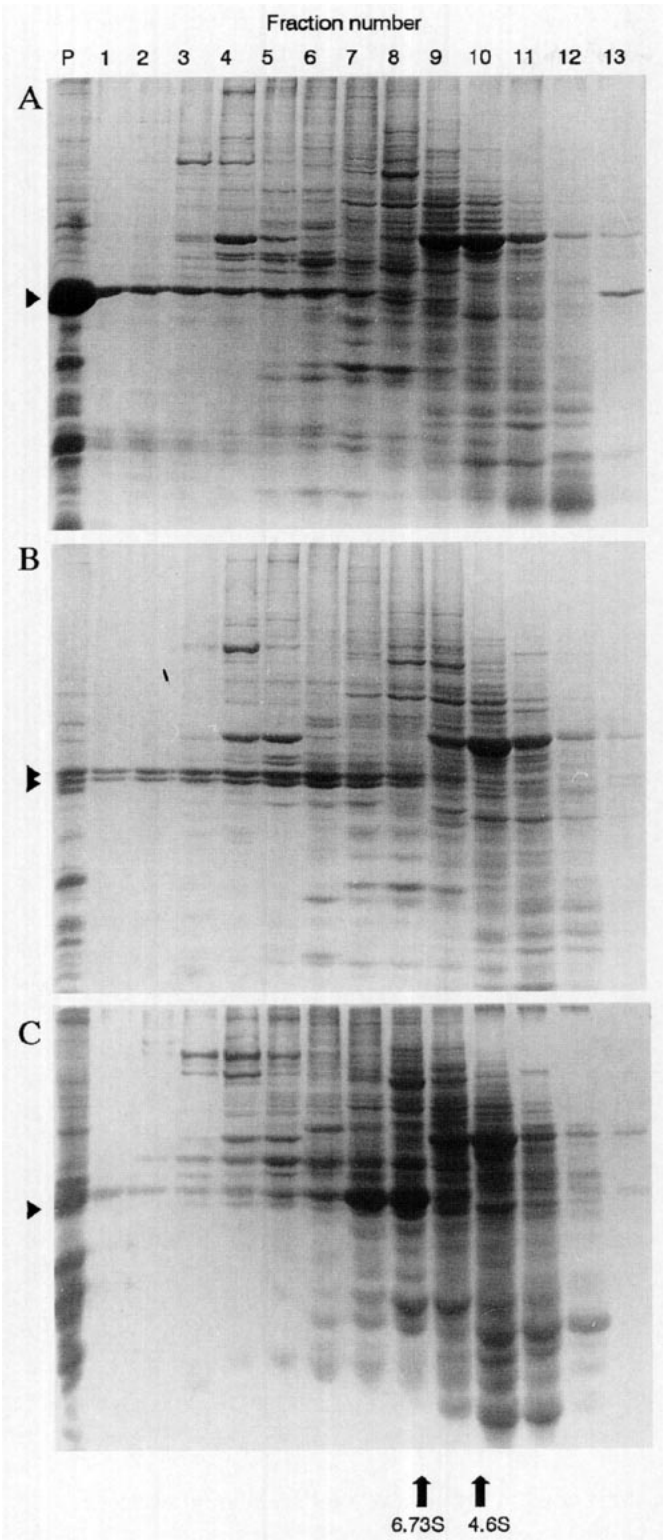

FIG. 1. Sedimentation analysis of NS2 expressed in baculovirus-infected cells. Cytoplasmic extracts were prepared from (A) AcBTV-10.8-, (B) AcEHDV-2.8-, and (C) AcAHSV-9.8-infected Sf9 cells in 0.15 *M* STE–TX and analyzed by sedimentation analysis on 10–40% sucrose gradients. The fractions and pellets (P) obtained following sedimentation analysis were analyzed by SDS–PAGE. Note that EHDV NS2 migrates as two distinct bands. The top of the gradients is to the right. The position of NS2 is indicated in each case, as well as the sedimentation peaks of bovine serum albumin (4.6S) and rabbit γ-globulin (6.73S).

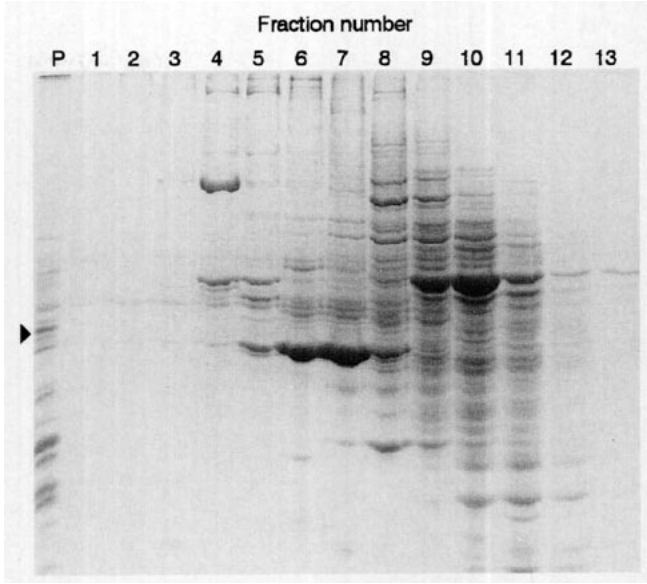

FIG. 2. Sedimentation analysis of NS2 expressed in baculovirus-infected cells following RNase treatment. A cytoplasmic extract of AcBTV-10.8-infected Sf9 cells was prepared, treated with RNase, and analyzed by sedimentation analysis on a 10–40% sucrose gradient. The fractions and pellet (P) were analyzed by SDS–PAGE. The top of the gradient is to the right. The position of NS2 is indicated.

or RNase were analyzed by SDS–PAGE and by immunoblot as shown in Fig. 3. In both cases, treatment with either 0.5 *M* NaCl or RNase prior to sedimentation analysis resulted in a shift in the sedimentation of NS2 toward the 7S position. The NS2 that was still present in the pellet after treatment is thought to be protein which has been trapped by insoluble cellular material. As was the case with baculovirus-expressed AHSV NS2, a large fraction of NS2 in AHSV-9-infected cells did not appear to be associated with ssRNA. These results suggested two characteristics of the NS2 protein of orbiviruses which were further investigated. First, the S value of unbound NS2 suggested that the protein is a multimer and second, the variant sedimentation behavior of BTV, AHSV, and EHDV NS2 proteins and its sensitivity to RNase treatment suggested that the three proteins differ in their ssRNA-binding ability.

NS2 is a multimer with disulfide bonds

Based on the sedimentation of unbound NS2 and the fact that NS2 is rich in cysteine residues (Hall *et al.*, 1989; Fukusho *et al.*, 1989; Van Staden *et al.*, 1991), the purified 7S NS2 complex, labeled with [^{35}S]methionine, was analyzed by reducing and nonreducing SDS–PAGE and immunoblot for the presence of intermolecular disulfide bonds (Fig. 4).

In the absence of reducing agent, SDS–PAGE analysis of NS2 revealed several novel higher molecular weight protein bands that were ^{35}S-labeled and reacted with NS2-monospecific antiserum in immunoblots. Based on these two lines of evidence, it was concluded that the

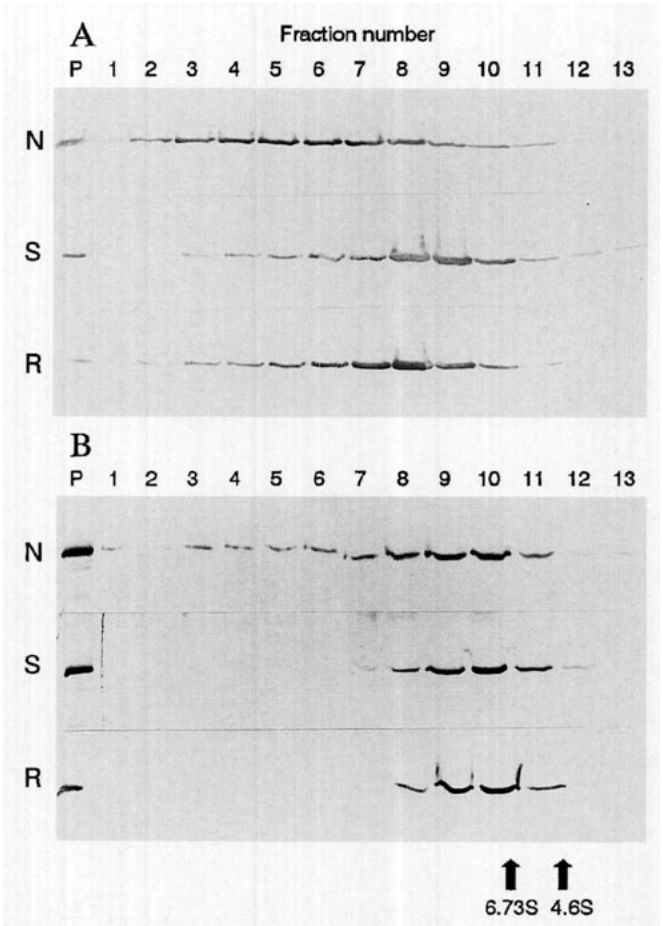

FIG. 3. Sedimentation analysis of NS2 expressed in orbivirus-infected cells following RNase or high-salt treatment. Cytoplasmic extracts were prepared from (A) BTV 10-infected BHK-21 cells and (B) AHSV-9-infected CER cells and analyzed by sedimentation analysis on 10–40% sucrose gradients following (N) no treatment, (S) treatment with 0.5 *M* NaCl prior to sedimentation analysis, and (R) treatment with RNase prior to sedimentation analysis. The fractions and (P) pellets obtained were analyzed by immunoblot. The sedimentation peaks of bovine serum albumin (4.6S) and rabbit γ-globulin (6.73S) are indicated.

higher molecular weight bands (labeled I, II, and III) are composed of multimers of NS2. Referring to unpublished data, Roy (1992) also found indications that BTV NS2 was multimeric. A ±97-kDa multimer (band I) of BTV NS2 (Fig. 4A) was found, which may be a dimer of the ±46-kDa NS2 monomer. NS2 of AHSV (Fig. 4B) and EHDV (Fig. 4C) yielded higher molecular weight bands of ≤200 kDa (band II), whereas ≥200-kDa bands (band III) were present in all three NS2 protein samples — BTV, EHDV, and AHSV NS2. Due to the anomalous migration of NS2 on SDS–PAGE gels, the exact number of NS2 monomers in the 7S multimer is not known. These bands may be tetramers (≤200 kDa) and hexamers or larger multimers (≥200 kDa). The band indicated by an asterisk on the immunoblot [Fig. 4A (ii)] is unreduced immunoglobulins bound by the protein A-conjugated secondary antibody. The relative intensity of bands I, II, and III differed from one repeat of the experiment to the next.

Under reducing conditions, NS2 expressed by AcBTV-10.8 and AcAHSV-9.8 migrated as a single band and NS2 of AcEHDV-2.8 migrated as two distinct bands. Under nonreducing conditions, all three NS2 proteins separated as two or three bands, the additional bands (indicated by ◁) migrating slightly faster than reduced NS2. These bands were also ^{35}S-labeled and reacted to monospecific anti-NS2 serum. The faster migration of these NS2 bands relative to reduced NS2 suggested that these proteins are more compact, due to unreduced intramolecular disulfide bonds. Kattoura *et al.* (1994) found similar results upon SDS–PAGE analysis of the rotavirus NSP2 (NS35) protein in the absence of reducing agent, following cross-linking of NSP2 with a thiol-cleavable cross-linking agent. NSP2 is known to have an intramolecular disulfide bond (Patton *et al.*, 1993). Both reduced and unreduced forms of monomer NSP2, as well as several unreduced multimer species of NSP2, were found.

Although very little monomeric NS2 was found by means of sedimentation analysis, most of the NS2 analyzed by SDS–PAGE in the absence of reducing agent electrophoresed as monomers with only small quantities of NS2 in the form of multimers. Mattion *et al.* (1992) and Poncet *et al.* (1993) found similar results upon nonreducing SDS–PAGE analysis of rotavirus NSP3. Monomer, dimer, and trimer or tetramer forms of NSP3 were found. This may be an indication that both NS2 and NSP3 are highly susceptible to endogenous reducing agents after denaturation by SDS. The finding that as little as 2 m*M* reducing agent was enough to dissociate the multimers of NSP3 supports this (Mattion *et al.*, 1992). It may, on the other hand, be an indication of different-sized multimers (Mattion *et al.*, 1992), although the sedimentation of NS2 as a homogenous 7S particle in nondenaturing sucrose gradients argues against it.

The NS2 proteins were also analyzed with or without boiling of the samples with no effect on the result (not shown), which indicated that hydrophobic interactions do not play an important role in maintaining the multimer. The above findings were subsequently confirmed by nonreducing SDS–PAGE of NS2 expressed by BTV and AHSV (results not shown). In summary, it was concluded that the structure of the NS2 multimer is maintained by both intermolecular and intramolecular disulfide bonds and that the multimer may contain six or more covalently bound NS2 monomers.

Differences in the *in vitro* ssRNA-binding abilities of AHSV, EHDV, and BTV NS2

It is known that BTV NS2 has an affinity for poly(U)–Sepharose (Huismans *et al.*, 1987a; Thomas *et al.*, 1990). In a preliminary experiment we found that sucrose-gradient-purified 7S NS2 complexes of BTV, AHSV, and EHDV are able to bind to poly(U)–Sepharose in the presence of low salt. This indicated that ssRNA-binding is a common feature of all three NS2 proteins and that the 7S multimeric form of NS2 is capable of ssRNA-binding.

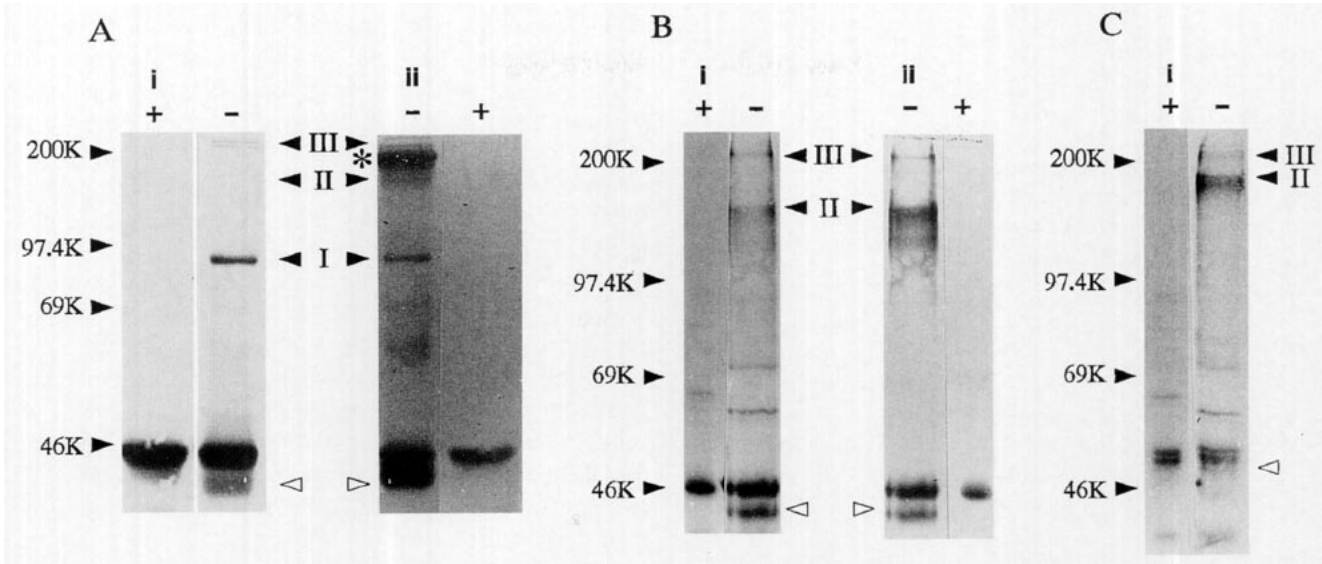

FIG. 4. Analysis of NS2 by SDS−PAGE in the presence (+) or absence (−) of β-mercaptoethanol as indicated. ^{35}S-Labeled NS2 was purified from (A) AcBTV-10.8-, (B) AcAHSV-9.8-, and (C) AcEHDV-2.8-infected Sf9 cell extracts and analyzed by (i) SDS−PAGE and autoradiography or (ii) immunoblot using monospecific serum against the appropriate NS2 protein. AcBTV-10.8 NS2 was purified by immunoprecipitation with anti-BTV NS2 serum, whereas AcAHSV-9.8 NS2 and AcEHDV-2.8 NS2 were purified by sedimentation analysis and poly(U)−Sepharose binding as described under Methods. The unreduced multimers are labeled I, II, and III and unreduced monomers are indicated by ◁. The band indicated by the asterisk is unreduced immunoglobulins. The positions and sizes of the molecular weight markers are shown.

To determine if NS2 of AHSV, EHDV, and BTV differs in its ability to bind ssRNA, we assayed binding of purified ^{35}S-labeled NS2 to poly(U)−Sepharose in the presence of increasing concentrations of NaCl. The results are shown in Fig. 5A. The ssRNA-binding abilities of the three proteins differed markedly. Only at the very lowest NaCl concentration (0.01 *M*) was the NS2 of all three orbiviruses quantitatively bound to the poly(U)−Sepharose, except for a small percentage (±5%) of AHSV NS2. Between 0.01 and 0.1 *M* NaCl there was a decline in the amount of bound NS2 in all three cases. However, in the case of BTV NS2, more than 70% was still bound at 0.2 *M* NaCl and a further decline in binding was only observed at 0.25 *M* NaCl. In the case of AHSV NS2, on the other hand, only about 45% was still bound at 0.1 *M* NaCl, and less than 20% was bound at a concentration of 0.2 *M* NaCl. The result obtained with EHDV NS2 was intermediate between those of BTV and AHSV. At both 0.15 and 0.2 *M* NaCl approximately 55% of the EHDV NS2 was bound, which steadily declined at higher salt concentrations. These results are also in agreement with the results obtained by sedimentation analysis of the ssRNA−NS2 complexes at 0.15 *M* STE−TX (Figs. 1 and 3).

Since poly(U)−Sepharose is a completely artificial substrate, the biological significance of this finding was unclear. Consequently the experiment was repeated using *in vitro*-transcribed ^{3}H-labeled BTV and AHSV mRNA and immunoprecipitation of the NS2−RNA complexes as described under Methods. mRNA-binding by BTV and AHSV NS2 was assayed in any combination of BTV and AHSV mRNA at the different salt concentrations indicated in Fig. 6. Irrespective of which mRNA was used, the RNA-binding efficiencies of the proteins to viral template were similar to their binding to poly(U)−Sepharose. From 0.1 to 0.3 *M* NaCl, BTV NS2 bound the viral mRNAs with greater efficiency than AHSV NS2 (Fig. 6). These results indicated that ssRNA-binding by NS2 is template nonspecific.

Subcellular localization of NS2

The marked difference between BTV and AHSV with respect to their ability to bind ssRNA at a physiological salt concentration raised the question whether this difference was also reflected in the subcellular location of AHSV and BTV NS2. No experiments with AHSV have as yet been carried out, but BTV NS2 is known to be associated with virus inclusion bodies in BTV-infected cells (Eaton *et al.*, 1987) and similar structures in Sf9 cells infected with a BTV NS2 baculovirus recombinant (Thomas *et al.*, 1990). Immunogold labeling of NS2 in AHSV-infected cells (Fig. 7A) indicated that the matrices of VIBs are composed mainly of NS2, as was previously found to be the case for BTV VIBs (Eaton *et al.*, 1987).

Even though no difference in the VIBs of BTV and AHSV was found, the possibility remained that in baculovirus-infected cells expressing AHSV NS2 the formation of the VIB-like structures would be affected by the difference in ssRNA-binding of AHSV and BTV NS2, due to the nonspecific nature of RNA-binding in the baculovirus-infected cell. Similar to baculovirus-expressed BTV NS2 (Fig. 8C), large granular bodies of NS2 that resembled the matrices of VIBs were found in the cytoplasm of AcAHSV-9.8-infected Sf9 cells (Figs. 7B and 8A). The NS2 bodies in AcBTV-10.8- (Fig. 8C) and AcAHSV-9.8-infected

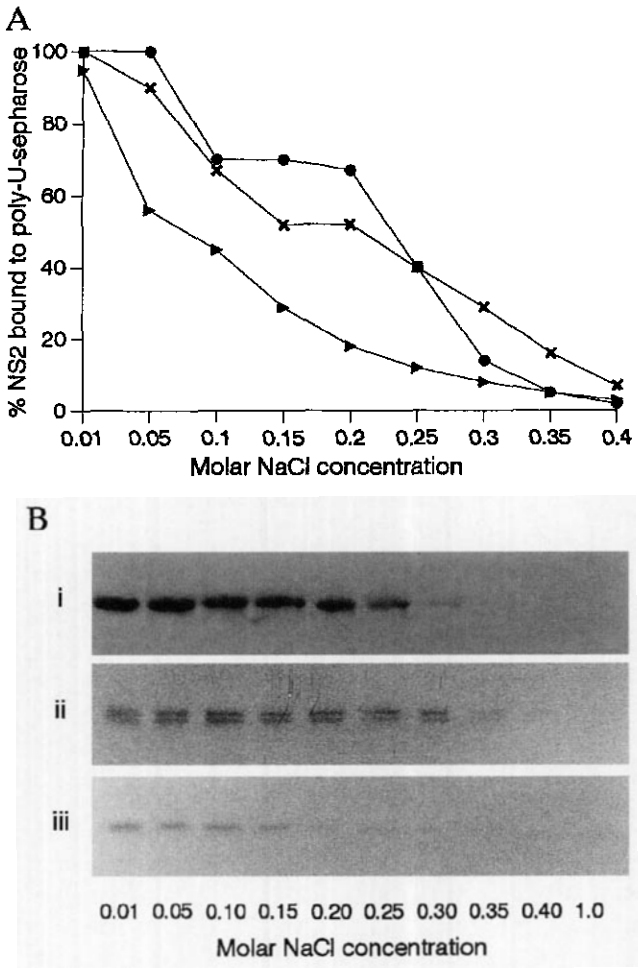

FIG. 5. Comparison of the ssRNA-binding by NS2 of BTV, AHSV, and EHDV in the presence of NaCl. NS2 was purified by sedimentation analysis of ^{35}S-labeled cytoplasmic extracts of Sf9 cells infected with one of the three NS2 baculovirus recombinants and ssRNA-binding was assayed by binding to poly(U)–Sepharose at various NaCl concentrations as described under Methods. The amount of NS2 bound to the poly(U)–Sepharose was quantitated by scintillation counting. (A) Graphic representation of the percentage of ^{35}S-labeled NS2 bound to poly(U)–Sepharose at different NaCl concentrations. AcBTV-10.8 NS2 (● —— ●), AcEHDV-2.8 NS2 (x —— x), AcAHSV-9.8 NS2 (▶ —— ▶). (B) Autoradiographs of the ^{35}S-labeled proteins bound to the poly(U)–Sepharose at the different NaCl concentrations, analyzed by SDS–PAGE. (i) AcBTV-10.8 NS2, (ii) AcEHDV-2.8 NS2, (iii) AcAHSV-9.8 NS2.

cells (Fig. 8A) were considerably larger compared to the VIBs found in BTV- (Fig. 8D) and AHSV-infected cells (Fig. 8B), probably due to the higher level of expression of NS2. The NS2 bodies in both recombinant baculovirus-infected cells were of no distinct size or shape. Similar results were found for NS2 expressed by AcEHDV-2.8 (not shown). These results indicated that the assembly of the NS2 into VIB-like structures in baculovirus-infected cells is not affected by the difference in affinity for ssRNA of BTV and AHSV NS2.

DISCUSSION

In this study we have shown that the NS2 proteins of three orbiviruses, AHSV, BTV, and EHDV, exist as multimers complexed with ssRNA in baculovirus-infected cells expressing NS2, as well as in cells infected with either BTV or AHSV. Sedimentation analysis indicated that very little monomeric NS2 is present and once dissociated from ssRNA by either RNase or high salt, NS2 sediments as a ±7S multimer. The ssRNA-binding capability of the multimers was demonstrated by their ability to bind to poly(U)–Sepharose.

SDS–PAGE analysis of NS2 in the absence of reducing agent indicated that the 7S NS2 multimer is a complex protein (≥200 kDa) containing several NS2 molecules covalently bound by inter- and intramolecular disulfide bonds. The sensitivity of SDS-denatured NS2 to reducing agent at low or high temperatures indicated that the structure of the multimer is highly dependent on disulfide bonds for stability. Each multimer appears to be composed of six or more covalently bound monomers. Although many factors other than molecular mass influence the sedimentation of a protein, this result was corroborated by the sedimentation of the 7S multimer in the region of gradients where proteins of MW ≥200 kDa sediment.

The nonstructural proteins σNS of reovirus and NSP3 of rotavirus have been shown to bind viral mRNA (Stamatos and Gomatos, 1982; Poncet *et al.*, 1993), as has been demonstrated for BTV NS2 (Huismans *et al.*, 1987a). σNS and NSP3 furthermore share a conserved motif with NS2 of AHSV, BTV, and EHDV (Van Staden *et al.*, 1991). It has therefore been proposed that NS2, NSP3, and σNS are functional equivalents (Poncet *et al.*, 1993). Since both NPS3 and σNS multimerize (Mattion *et al.*, 1992; Gomatos *et al.*, 1980), the fact that NS2 has now also been shown to multimerize lends further support to the proposal.

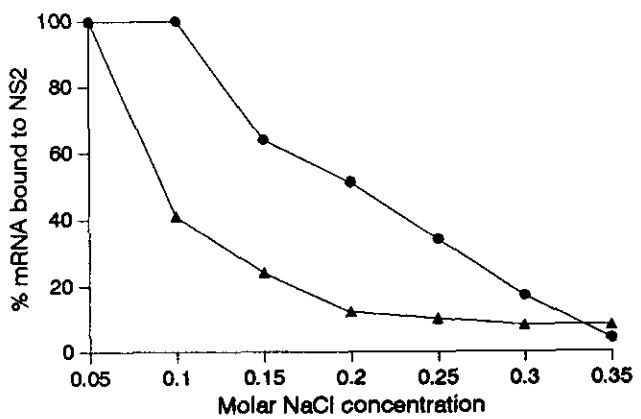

FIG. 6. Comparison of the ssRNA-binding by NS2 of BTV and AHSV at different NaCl concentrations. NS2 was purified by sedimentation analysis of ^{35}S-labeled cytoplasmic extracts of Sf9 cells infected with AcBTV-10.8 or AcAHSV-9.8 and ssRNA-binding was assayed by binding to *in vitro*-transcribed ^{3}H-labeled BTV or AHSV mRNA as described under Methods. The NS2–mRNA complexes were immunoprecipitated and the percentage mRNA bound to NS2 was quantitated by scintillation counting. AcBTV-10.8 NS2 (● —— ●) and AcAHSV-9.8 NS2 (▶ —— ▶).

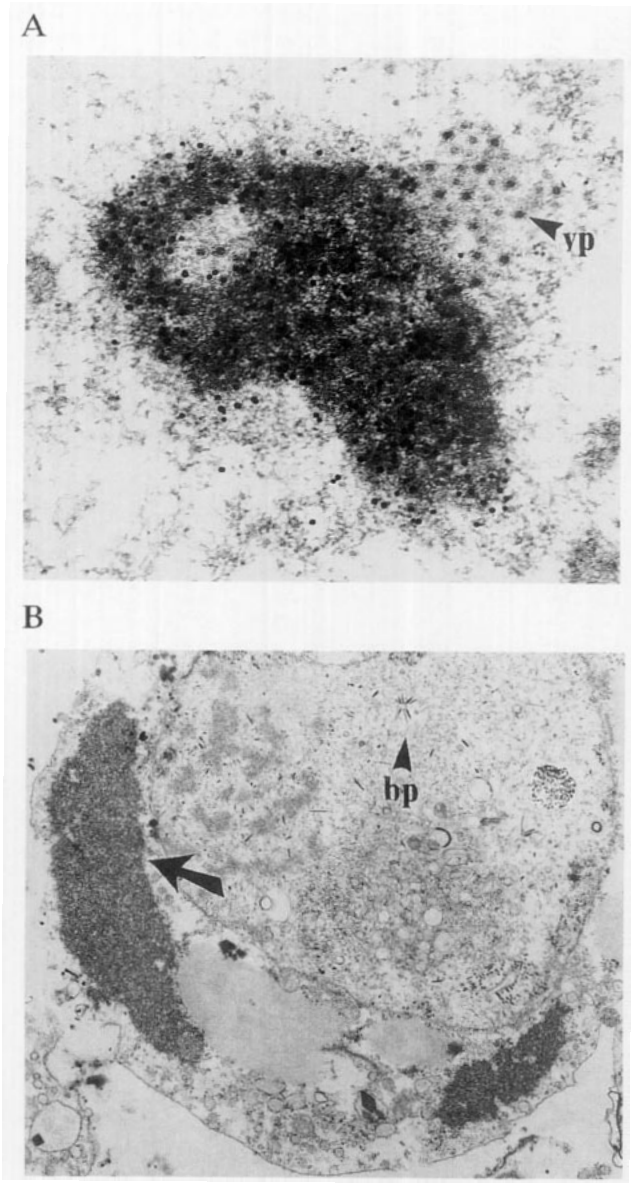

FIG. 7. Electron microscopic investigation of NS2-containing structures in infected cells. (A) Virus inclusion body (VIB) from an AHSV-9-infected Vero cell, immunogold-labeled with monospecific anti-AHSV NS2 serum; vp, AHSV-9 virus particle. (B) A granular body of NS2 (indicated by arrow) formed in an AcAHSV-9.8-infected Sf9 cell; bp, baculovirus particles.

Distinct differences in the ability of NS2 of BTV, EHDV, and AHSV to bind nonspecifically to ssRNA were found over a wide range of NaCl concentrations. The variant ssRNA-binding of the three NS2 proteins, however, did not affect the tendency of the proteins to accumulate in VIB-like structures in baculovirus-infected cells. NS2 distinguishes itself from rotavirus NSP3 and reovirus σNS in that it is phosphorylated (Huismans and Basson, 1983). Phosphorylation of proteins frequently regulates nucleic acid binding (Green *et al.*, 1992). It has been shown that baculovirus-expressed BTV, AHSV, and EHDV NS2 differ in the degree to which they can be phosphorylated *in vitro* (Theron *et al.*, 1994). It was also shown that phosphorylation of unphosphorylated bacterially expressed EHDV NS2 resulted in some inhibition of ssRNA-binding at low-salt concentrations. The difference in ssRNA-binding of NS2 of the three orbiviruses could therefore be a result of varying degrees of phosphorylation of the respective proteins.

The disparity in ssRNA-binding may also be a result of structural differences in the three NS2 proteins. Although these proteins do not have a recognized ssRNA-binding motif, a conserved feature is their high α-helix content (Van Staden *et al.*, 1991). This has also been reported for reovirus σNS (Wiener and Joklik, 1987) and rotavirus NSP3 (Both *et al.*, 1984). It has been suggested in the case of reovirus σNS and the matrix protein M1 of influenza A virus that these viral proteins interact with RNA via α-helices (Richardson and Furuichi, 1983; Wakefield and Brownlee, 1989). If RNA-binding is associated with the α-helix-rich C-terminal of NS2, it is noteworthy that, based on secondary structure predictions, BTV NS2 has the highest α-helix content (69%), followed by EHDV NS2 (59%), and AHSV NS2 (47%) (Huismans *et al.*, 1991). These results suggest a correlation between the α-helix content of the NS2 protein and the nonspecific ssRNA-binding ability of the protein. Support for this has come from an investigation into the ssRNA-binding of bacterially expressed NS2 of EHDV and AHSV. Preliminary results seem to indicate that, as is the case with the baculovirus-expressed protein, AHSV NS2 binds ssRNA with a

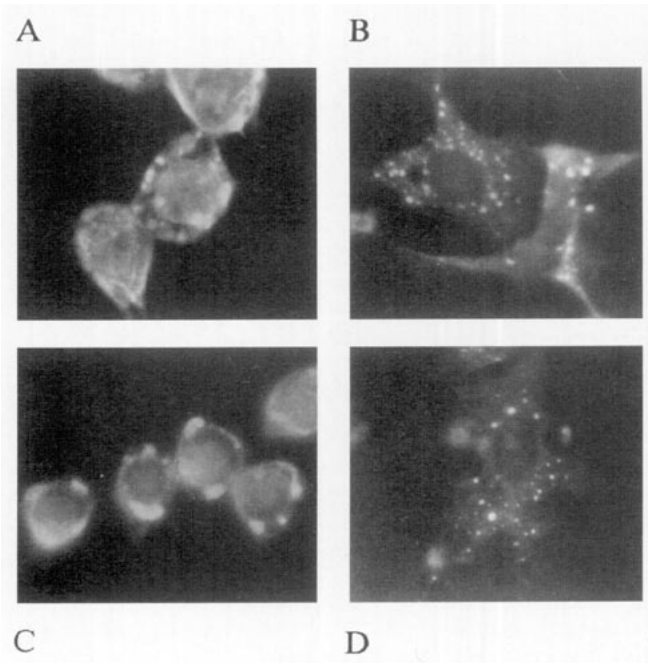

FIG. 8. Comparison of the NS2-containing structures found in infected cells by means of indirect immunofluorescence. Localization of AHSV NS2 in (A) AcAHSV-9.8-infected Sf9 cells and in (B) AHSV-9-infected Vero cells by labeling with monospecific anti-AHSV NS2 serum. Localization of BTV NS2 in (C) AcBTV-10.8-infected Sf9 cells and in (D) BTV-10-infected Vero cells by labeling with monospecific anti-BTV NS2 serum.

much lower efficiency at physiological salt concentration than EHDV NS2.

It still remains to be determined if any specific interaction between NS2 and viral mRNA occurs. The results described here do not rule out sequence-specific RNA-binding by NS2. However, should binding specificity be present it would be masked by the nonspecific interaction of NS2 with any ssRNA template. It has been shown that both σNS of reovirus and NSP3 of rotavirus bind the 3′ ends of viral mRNA (Stamatos and Gomatos, 1982; Poncet *et al.*, 1993). Huismans *et al.* (1987a) found that BTV NS2 binds viral mRNA with the formation of a 22S complex pointing to some form of specificity in NS2–mRNA-binding. No association with the 3′ end of viral mRNA has, however, been demonstrated for NS2 as yet. If NS2 is indeed involved in the selection of the 10 different segments some specificity with viral mRNA will have to be demonstrated.

ACKNOWLEDGMENTS

We thank Vida van Staden and Louis Nel for assistance with the preparation of the manuscript, as well as Alan Hall for assistance with the electron microscopy. This research was made possible by financial support from the Foundation for Research Development.

REFERENCES

Both, G. W., Bellamy, A. R., and Siegman, L. J. (1984). Nucleotide sequence of the dsRNA genomic segment 7 of simian 11 rotavirus. *Nucleic Acids Res.* **12,** 1621–1626.

Boyle, J. F., and Holmes, K. V. (1986). RNA-binding proteins of bovine rotavirus. *J. Virol.* **58,** 561–568.

Brookes, S. M., Hyatt, A. D., and Eaton, B. T. (1993). Characterization of virus inclusion bodies in bluetongue virus-infected cells. *J. Gen. Virol.* **74,** 525–530.

Eaton, B. T., Hyatt, A. D., and White, J. R. (1987). Association of bluetongue virus with the cytoskeleton. *Virology* **157,** 107–116.

Fukusho, A., Yu, Y., Yamaguchi, S., and Roy, P. (1989). Completion of the sequence of bluetongue virus serotype 10 by the characterization of a structural protein VP6, and a non-structural protein NS2. *J. Gen. Virol.* **10,** 1766–1689.

Gomatos, P. J., Prakash, O., and Stamatos, N. M. (1980). Small reovirus particles composed solely of sigma NS with specificity for binding different nucleic acids. *J. Virol.* **39,** 115–124.

Green, P. L., Yip, M. T., Xie, Y., and Chen, I. S. Y. (1992). Phosphorylation regulates RNA binding by the human T-cell leukemia virus Rex protein. *J. Virol.* **66,** 4325–4330.

Hall, S. J., Van Dijk, A. A., and Huismans, H. (1989). Complete nucleotide sequence of gene segment 8 encoding non-structural protein NS2 of SA bluetongue virus serotype 10. *Nuclei Acids Res.* **17,** 457.

Huismans, H., and Basson, H. M. (1983). The characterization of a phosphorylated non-structural polypeptide of BTV with affinity for RNA. *In* "Double-Stranded RNA Viruses" (R. W. Compans and D. H. L. Bishop, Eds.). Elsevier, New York.

Huismans, H., and Joklik, W. K. (1976). Reovirus-coded polypeptides in infected cells: Isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively. *Virology* **70,** 411–424.

Huismans, H., Van Dijk, A. A., and Bauskin, A. R. (1987a). In vitro phosphorylation and purification of a non-structural protein of bluetongue virus with affinity for single-stranded RNA. *J. Virol.* **61,** 3589–3595.

Huismans, H., Van Dijk, A. A., and Els, H. J. (1987b). Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. *Virology* **157,** 180–188.

Huismans, H., Van Staden, V., and Nel, L. H. (1991). Molecular comparison of the cognate genes of bluetongue, African horse sickness, and epizootic hemorrhagic disease viruses which encode non-structural proteins NS2 and NS3. *In* "Bluetongue, African Horse Sickness and Related Orbiviruses." Proc. Second Int. Symp. (T. E. Walton and B. I. Osburn, Eds.). CRC Press, Boca Raton, FL.

Kattoura, M. D., Chen, X., and Patton, J. T. (1994). The rotavirus RNA-binding protein NS35 (NSP2) forms 10S multimers and interacts with the viral RNA polymerase. *Virology* **202,** 803–813.

Laemmli, U. K. (1970). Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature* **227,** 680–685.

Lecatsas, G. (1968). Electron microscopic study of the formation of bluetongue virus. *Onderstepoort J. Vet. Res.* **35,** 139–150.

Mattion, N. M., Cohen, J., Aponte, C., and Estes, M. K. (1992). Characterization of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34. *Virology* **190,** 68–83.

Patton, J. T., Salter-Cid, L., Kalbach, A., Mansell, E. A., and Kattoura, M. (1993). Nucleotide and amino acid sequence analysis of the rotavirus nonstructural RNA-binding protein, NS35. *Virology* **192,** 438–446.

Poncet, D., Aponte, C., and Cohen, J. (1993). Rotavirus protein NSP3 (NS34) is bound to the 3′ end consensus sequence of viral mRNAs in infected cells. *J. Virol.* **67,** 3159–3165.

Richardson, M. A., and Furuichi, Y. (1983). Nucleotide sequence of reovirus genome segment S3, encoding non-structural protein sigma NS. *Nucleic Acids Res.* **11,** 6399–6408.

Roy, P. (1992). Bluetongue virus proteins. *J. Gen. Virol.* **73,** 3051–3064.

Stamatos, N. M., and Gomatos, P. J. (1982). Binding to select regions of reovirus mRNAs by a non-structural reovirus protein. *Proc. Natl. Acad. Sci. USA* **79,** 3457–3461.

Summers, M. D., and Smith, G. E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric. Exp. Stn. Bull.* **1555.**

Theron, J., Uitenweerde, J. M., Huismans, H., and Nel, L. H. (1994). Comparison of the expression and phosphorylation of the non-structural protein NS2 of three different orbiviruses: Evidence that phosphorylation modulates ssRNA-binding and involves an ubiquitous cellular kinase. *J. Gen. Virol.* **75,** 3401–3411.

Thomas, C. P., Booth, T. F., and Roy, P. (1990). Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: It binds the single-stranded RNA species. *J. Gen. Virol.* **71,** 2073–2083.

Van Dijk, A. A., and Huismans, H. (1988). *In vitro* transcription and translation of bluetongue virus mRNA. *J. Gen. Virol.* **69,** 573–581.

Van Staden, V., Theron, J., Greyling, B. J., Huismans, H., and Nel, L. H. (1991). A comparison of the nucleotide sequences of cognate NS2 genes of three different orbiviruses. *Virology* **185,** 500–504.

Wakefield, L., and Brownlee, G. G. (1989). RNA-binding properties of influenza A virus matrix protein M1. *Nucleic Acids Res.* **17,** 8569–8580.

Wiener, J. R., and Joklik, W. K. (1987). Comparison of the reovirus serotype 1, 2, and 3 S3 genome segments encoding the non-structural protein σNS. *Virology* **161,** 332–339.