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Deletion and Mutational Analyses of Bluetongue Virus NS2 Protein Indicate that the Amino but Not the Carboxy Terminus of the Protein Is Critical for RNA-Protein Interactions

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Genome segment 8 (S8) of bluetongue virus serotype 10 (BTV-10) encodes the nonstructural protein NS2. This protein, which has single-stranded RNA (ssRNA) binding capacity, is found in BTV-infected cells in the form of virus inclusion bodies (VIBs). To identify the domain(s) important for RNA binding and oligomerization of the protein, a number of deletions were made in regions of the gene that code for either the amino or carboxy terminus of the protein. The modified genes were cloned into and expressed from baculovirus vectors based on *Autographa californica* nuclear polyhedrosis virus. Truncated NS2 proteins were individually analyzed for the ability to bind ssRNA and to form VIBs. The results indicated that the carboxy terminus of the protein is involved neither in RNA binding nor in the formation of VIBs. The amino terminus of NS2 was shown to be essential for ssRNA binding but not for NS2 protein oligomerization. Point mutations that involved the substitution of various charged residues at the amino terminus of NS2 were generated and tested for the ability to bind ssRNA. The results showed that the arginines at amino acid residues 6 and 7 and the lysine at residue 4, but not the glutamic acid at residue 2, are involved in ssRNA binding.

In infected cells, certain members of the family *Reoviridae* (reoviruses, the rotaviruses, orbiviruses, etc.) synthesize a number of nonstructural (NS) proteins, in addition to the structural proteins of the respective viruses. Although the functions of the NS proteins are poorly understood, it is believed that they are involved in the processes of virus replication and morphogenesis, leading to virion assembly and release. Virion assembly involves the intracellular accumulation of viral genes and structural proteins and the inclusion of both components into subviral structures, leading eventually to the formation of mature virions. For orbiviruses such as bluetongue virus (BTV), the genome consists of 10 double-stranded RNA species (L1 to L3, M4 to M6, and S7 to S10). It is believed that the virus mRNA species are the precursors of the virion genes, although in what form or how these and other virion components are assembled to make virus particles is not known.

The 357-amino-acid, 41-kDa NS2 protein, the product of the BTV serotype 10 (BTV-10) S8 gene, has been shown to possess single-stranded RNA (ssRNA) binding capacity (3, 8, 20). NS2 does not bind double-stranded RNA. NS2 forms virus inclusion bodies (VIBs) in BTV-infected mammalian cells and in insect cells, using baculovirus vectors, when expressed in the absence of other BTV genes (20). This property is similar to that of the rotavirus NS35 protein, which also forms oligomeric structures, binds ssRNA, and exists in complexes with viral RNA species in infected cells (9). The BTV NS2 protein has seven cysteine residues, four of which are located near the carboxy terminus (6). The protein is generally hydrophilic, with pairs of positively charged amino acids (R) located near both ends of the protein.

To investigate the functional attributes of NS2, we have sought to identify regions of the protein that are involved in ssRNA binding and/or protein oligomerization. A number of truncated NS2 proteins, as well as single amino acid mutations, were derived. Using baculovirus vectors, we examined each deletion derivative for its ability to form oligomers. The deletion derivatives and point mutants were also used in ssRNA binding experiments. The data indicate that the carboxy terminus of NS2 (including the last 130 amino acids) is not required for ssRNA binding or for oligomerization. While the amino terminus of NS2 (up to residue 92) was found not to be required for oligomerization, certain amino acids located at the amino terminus, i.e., the arginines at residues 6 and 7 and a lysine at residue 4, but not a glutamic acid at residue 2, were required for ssRNA binding.

MATERIALS AND METHODS

Viruses and cells. *Autographa californica* nuclear polyhedrosis virus and derived recombinant viruses were grown and assayed in suspension or monolayer cultures of *Spodoptera frugiperda* cells in medium containing 5 to 10% (vol/vol) fetal calf serum as described by Brown and Faulkner (1).

DNA manipulations and construction of recombinant transfer vectors. The recombinant plasmid pAcBTV-10.8 (20) was used to prepare NS2 derivatives with carboxy-terminal deletions. The sequence containing the entire coding region of NS2 was recovered by *Bam*HI digestion and treated with either *Xba*I or *Hin*dIII, and the DNA was repaired with the Klenow fragment of DNA polymerase and cloned into the *Nhe*I site of the baculovirus transfer vector pJVP10Z (21). Recombinant plasmids were recovered and were designated pAcNS2ΔC130 and pAcNS2ΔC49, respectively. The correct orientation of the gene in each transfer vector was verified by sequence analysis

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TABLE 1. Sequences of PCR primers used for constructing NS2 mutants

Type of primer	Mutation	Sequence
Forward	ΔN8	5′-GCGCGAGCTCCCATGAAAAACATTTTTGTCTTGGAC-3′
	ΔN18	5′-GCGCGAGCTCCCATGAAAACATTATGCGGGGCTATC-3′
	ΔN25	5′-GCGCGAGCTCCCATGAAGTTGAGTTCGCAGCCGTATTGT-3′
	K4L	5′-GCGCGAGCTCCCATGGAGCAATTGCAACGTAGA-3′
	R6L	5′-GCGCGAGCTCCCATGGAGCAAAAGCAACTTAGATTTACC-3′
	R7L	5′-GCGCGAGCTCCCATGGAGCAAAAGCAACGTTTATTTACC-3′
	E2L	5′-GCGCGAGCTCCCATGCTGCAAAAGCAACGT-3′
Reverse		5′-CAAGCTAATGATATCCTGCCC-3′

(18). For deletions or point mutations at the amino terminus of the NS2, PCRs were used to introduce a *Sac*I linker and an ATG start codon at the 5′ end of the coding region and a *Pst*I site at the 3′ end. Pairs of forward and reverse primers were synthesized for each construction. The sequences of these primers are shown in Table 1. PCR mixtures contained 5 μl of 10× *Taq* polymerase buffer, 100 ng of forward primer, 100 ng of reverse primer, 0.5 μl of pUC4.BTV-10.8 (50 ng) (20), 1 U of *Taq* polymerase, and 5 μl of 2.5 mM deoxynucleoside triphosphate mix in a total volume of 50 μl. The PCR program involved 5 cycles of 96°C (1 min), 40°C (30 s), 50°C (30 s), and 72°C (1 min), followed by 30 cycles of 96°C (1 min), 50°C (1 min), and 72°C (1 min) and 1 cycle of 72°C (5 min). The PCR products were treated with proteinase K for 30 min at 37°C, recovered, then digested with *Bam*HI or with *Sac*I and *Pst*I, and cloned into *Bam*HI-*Nhe*I-cut pJVP10Z or *Sac*I-*Pst*I-cut pAcCL29 for the C-terminal truncations or N-terminal modifications, respectively. Recombinant vectors were designated pAcNS2ΔN92, pAcNS2ΔN25, pAcNS2ΔN18, pAcNS2ΔN8, pAcNS2K4L, pAcNS2R6L, pAcNS2R7L, and pAcNS2E2L to indicate the positions and lengths of the deleted sequences or the amino acids that were mutated (K, R, E), their positions, and substitution by L (see text).

Transfection and selection of recombinant baculoviruses. Recombinant transfer vectors were cotransfected into *S. frugiperda* cells in the presence of purified *Bsu*361-cut AcRP6-SC DNA (11) in order to generate recombinant baculoviruses. These viruses were plaque purified two to three times, and isolated plaques were used to infect *S. frugiperda* cells in order to generate recombinant virus stocks. The viruses corresponding to the recombinant transfer vectors listed above were designated AcNS2ΔC130, AcNS2ΔC49, AcNS2ΔN92, AcNS2ΔN25, AcNS2ΔN18, AcNS2ΔN8, AcNS2K4L, AcNS2R6L, AcNS2R7L, and AcNS2E2L, respectively.

SDS-PAGE and Western immunoblot analyses of expressed proteins. Infected cells were harvested at 48 or 72 h postinfection (p.i.), depending on the cytopathic effect, washed in phosphate-buffered saline, and then lysed in 0.01 M Tris-HCl buffer (pH 7.6). An equal volume of protein dissociation buffer (13) was added, and the samples were heated for 10 min at 100°C. Samples of 15 μl were resolved by polyacrylamide gel electrophoresis (PAGE) on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (13). Proteins were subsequently detected by Coomassie brilliant blue staining. Western blot analysis was performed as described by French et al. (5), with minor modifications. The antiserum used (1:1,000 dilution) was a rabbit anti-BTV-10 serum (5) or a guinea pig antiserum raised against a suspension of NS2 in 0.85% NaCl. This suspension consisted of baculovirus-expressed NS2 excised from an SDS–10% polyacrylamide gel after identification by 0.3 M KCl precipitation (20). For Western analyses, the bound guinea pig serum was reacted with goat anti-guinea pig immunoglobulin G conjugated to alkaline phosphatase prior to treatment with an alkaline phosphatase substrate.

Poly(U)-Sepharose 4B affinity chromatography. Supernatants of infected cell lysates were applied to 4-ml columns of poly(U)-Sepharose 4B (Sigma). The bound protein samples were eluted with linear gradients of 0.15 to 1.15 M NaCl in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA [pH 7.4]), and the products were analyzed by Western blotting as described by Thomas et al. (20).

Preparation of ssRNA probes. ^{32}P-labelled BTV segment S10 ssRNA was synthesized in vitro, using a Promega transcript kit according to the manufacturer's instructions. A pGEM-3zf(+) vector containing the S10 gene (23) under the control of the T7 promoter was used to produce ssRNA transcripts. The vector was linearized with *Hin*dIII prior to production of 1.08-kb transcripts, using T7 polymerase. Five units of DNase (Pharmacia) was added to destroy the DNA template after the transcription reactions were completed. RNA transcripts were purified through Sepharose CL-6B columns and stored as 50-μl samples at −70°C.

Retardation assays for RNA binding. Expressed NS2 proteins were partially purified by precipitation with 40% (wt/vol) ammonium sulfate and then desalted through a Sephadex G-25M (Pharmacia) desalting column. Protein samples (2.5 μg) were incubated in binding buffer [2 mM $MgCl_2$, 60 mM KCl, 150 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 100 mM dithiothreitol, 1 mM EDTA, 10% glycine, 10% glycerol, 5 U of RNA-Guard, 1 U of poly(dI-dC) (Pharmacia)] at room temperature for 15 min. ^{32}P-labelled ssRNA (1 μg, 10^5 cpm) was added to each reaction mixture, and incubation continued at 18°C for 10 min. The RNA-protein complexes were resolved by electrophoresis on a 0.8% agarose gel containing 50 mM glycine, 50 mM Tris (pH 8.0), and 0.1% sodium deoxycholate (12). Electrophoresis was performed at 60 V until the bromophenol blue dye front was 15 to 20 mm from the bottom of the gel. The wet gels were fixed with 10% methanol–10% acetic acid for 15 min and dried on a vacuum drying block system prior to autoradiography at −70°C.

Electron microscopy. Recombinant or wild-type virus-infected *S. frugiperda* cells were harvested at 24 h p.i., fixed in 1% paraformaldehyde, then dehydrated, embedded, and sectioned as described previously (20). For conventional electron microscopy, the cells were treated with osmium tetroxide before the dehydration steps. Final staining of the grids was accomplished by using uranyl acetate and lead citrate. Sections of cells on grids were analyzed for NS2 antigen by using immunogold techniques (20). The primary antiserum used was anti-BTV-10 rabbit serum (1:150 dilution). The second antiserum was goat

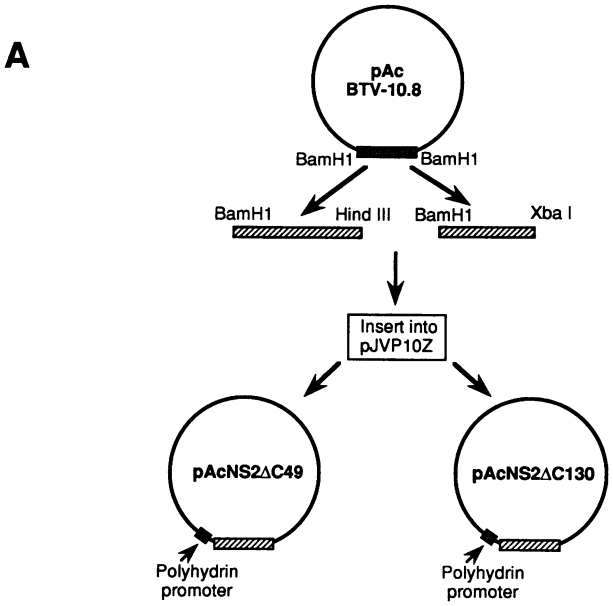

FIG. 1. NS2 deletion mutants used in these analyses. (A) Cloning strategy used for preparation of the pAcNS2ΔC49 and pAcNS2ΔC130 transfer vectors (see Materials and Methods). (B) Schematics of the carboxy- and amino-terminal deletions of NS2 that were produced.

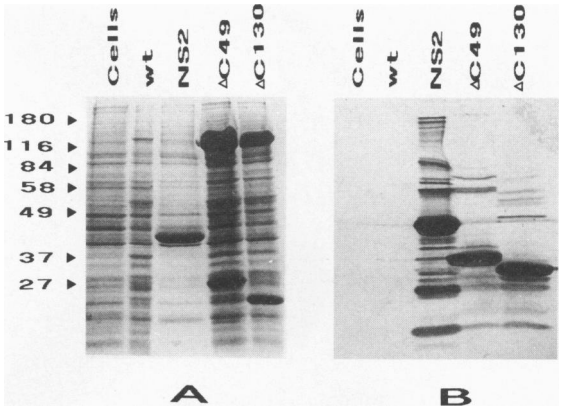

FIG. 2. Expression of carboxyl-terminal truncated mutants of NS2. Cell extracts were prepared 24 h p.i. and resolved by SDS-PAGE prior to staining with Coomassie brilliant blue (A) or Western blot analyses (B) as described in Materials and Methods. Uninfected cells (Cells) and AcRP6-SC-infected cells (wild type [wt]) were processed similarly. The recombinants AcNS2ΔC49 (ΔC49) and AcNS2ΔC130 (ΔC130) expressed β-galactosidase in addition to the mutant NS2 proteins. The positions of molecular weight markers are shown in kilodaltons on the left.

anti-rabbit immunoglobulin G conjugated to 10-nm colloidal gold particles.

RESULTS

The carboxy terminus of NS2 is not involved in ssRNA binding or in the formation of VIBs. To investigate whether the carboxy-terminal region of BTV NS2 protein was required for VIB formation or ssRNA binding, derivatives of the BTV-10 S8 gene lacking the carboxy-terminal 130 or 49 amino acids of NS2 were prepared as described in Materials and Methods and illustrated in Fig. 1A. The 49-amino-acid deletion resulted in the removal of four of the seven cysteine residues of the protein as well as three regions where pairs of basic amino acids are present (6). In addition to this, the 130-amino-acid deletion resulted in the removal of one region of the protein that is rich in basic amino acids and another that is rich in acidic amino acids (6). The two derivatives were cloned into the pJVP10Z transfer vector to allow the identification of recombinant viruses (AcNS2ΔC49 and AcNS2Δ C130) through the concomitant expression of β-galactosidase. *S. frugiperda* cell monolayers were mock infected or infected with the virus AcRP6-SC, AcBTV-10.8, AcNS2ΔC49, or AcNS2ΔC130 and harvested at 24 h p.i. Cell extracts were resolved by SDS-PAGE, stained with Coomassie brilliant blue (Fig. 2A), or used for Western blot analyses (Fig. 2B). By comparison with the 41-kDa unmodified NS2 protein, the recombinants AcNS2ΔC49 and AcNS2ΔC130 formed proteins that were estimated to be ca. 36 and 25 kDa in size (Fig. 2A; designated ΔC49 and ΔC130, respectively). They reacted with anti-BTV-10 rabbit serum in Western blot analyses (Fig. 2B). Although some degradation products were identified in the Western analyses of the unmodified NS2 extracts, these were considered to be minor components in view of the stained gel results (Fig. 2A). Between experiments, the amounts of such products varied.

To investigate whether the NS2 deletion derivatives were able to form VIBs in insect cells, conventional and immunogold electron microscopy was used. For both viruses with deleted NS2 sequences, we identified VIBs that reacted with gold-tagged BTV antibodies (Fig. 3) but not with antiserum raised with BTV NS2 protein (data not shown). To determine whether the two carboxy-terminal truncated NS2 proteins bound ssRNA, a poly(U)-Sepharose 4B chromatography system was used (Materials and Methods). Both proteins bound to the substrate and were eluted at salt concentrations in excess of 0.4 M NaCl (Fig. 4, filled arrowheads). Depending on the experiment, polymeric forms of the proteins were also recovered (Fig. 4B, open arrowhead). The origins of these polymers were not investigated. In addition, in this analysis, a number of NS2 antibody-reactive degradation products were identified in the materials applied to the columns. For example, with the 130-amino-acid deletion mutant, at least six such products were observed (Fig. 4B, lane C). Four of these products were eluted by the salt gradient. Some exhibited slightly different elution properties by comparison to the intact protein. Two species appeared not bind to the substrate or to be recovered during the gradient elution, indicating that some regions of the protein did not bind to an RNA substrate. The relationships of these proteins to the intact protein or to those that bound to the support were not investigated further.

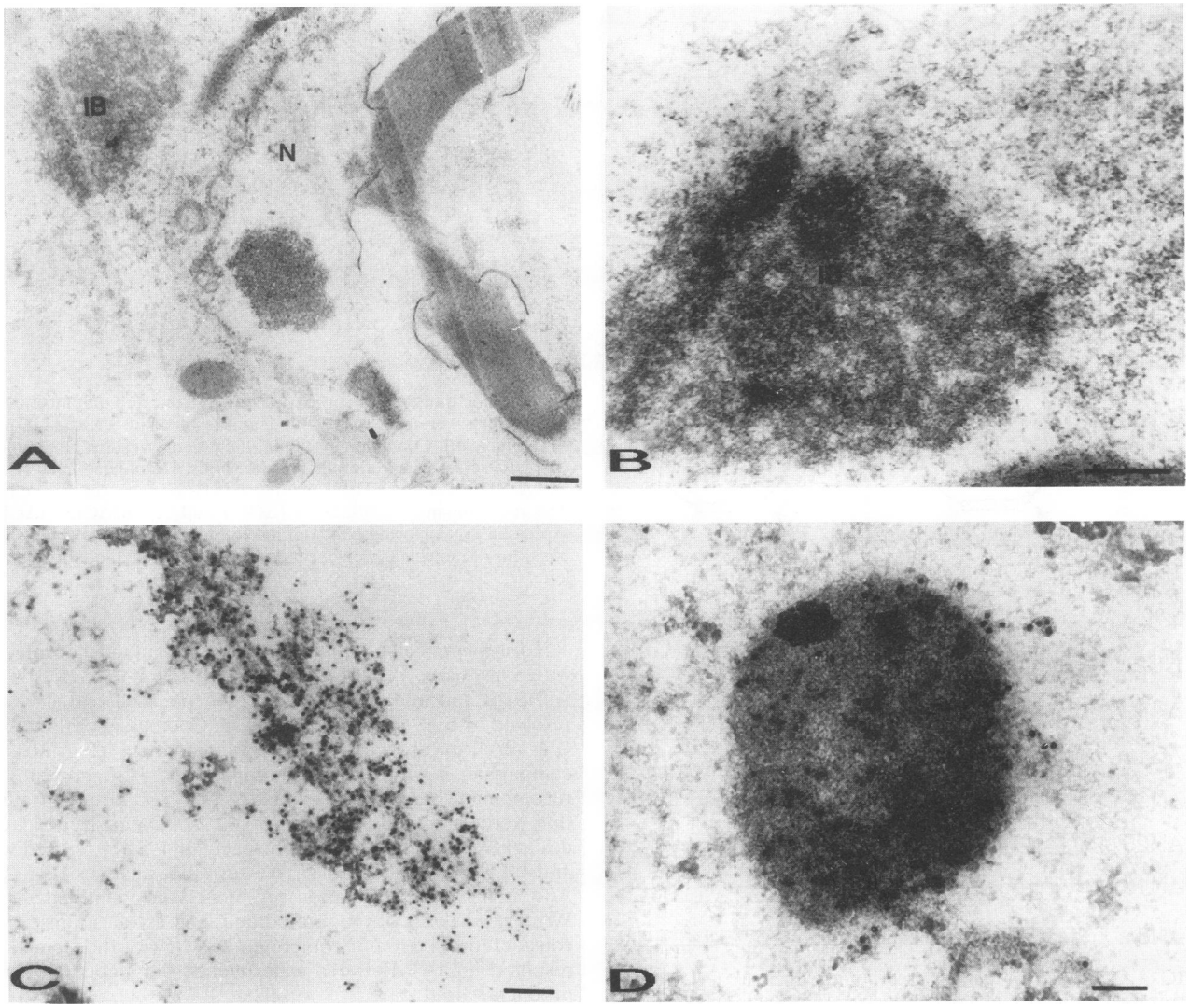

FIG. 3. Electron micrographs of *S. frugiperda* cells infected with recombinant virus AcNS2ΔC49 (A and C) or AcNS2ΔC130 (B and D). *S. frugiperda* cells were infected with the viruses and harvested 24 h p.i. They were fixed, treated with osmium tetroxide, dehydrated, embedded, sectioned, and transferred to grids as described in Materials and Methods. Sections were stained with uranyl acetate and lead citrate. (A and B) Conventional electron micrographs (IB, inclusion body; N, nucleus); (C and D) immunoelectron micrographs. For the latter, sections of cells on the grids were incubated with a 1:150 dilution of anti-BTV-10 rabbit serum and then with 10-nm colloidal gold conjugated to goat anti-rabbit immunoglobulin G (1:50 dilution) and subsequently stained with uranyl acetate. Bars: 1 μm (A), 500 nm (B), 150 nm (C), and 100 nm (D).

The N terminus of NS2 is required for ssRNA binding. To determine whether the amino terminus of NS2 is involved in ssRNA binding, four deletion mutants of the gene (NS2ΔN8, NS2ΔN18, NS2ΔN25, and NS2ΔN92) were generated as described in Materials and Methods (Fig. 1B). The smallest deletion removed the amino terminus that has the sequence (M)EQKQRRF... In addition to this, the 25-amino-acid deletion removed a hydrophobic domain and one of the cysteine residues of NS2, while the largest deletion removed another cysteine. The mutants were introduced into pAcCL29 transfer vectors (i.e., vectors lacking the β-galactosidase gene) and used to make expression vectors. The synthesis of each truncated protein in *S. frugiperda* cells was confirmed by SDS-PAGE and by Western blot analysis using anti-BTV NS2 serum (Fig. 5). The relative mobilities of the truncated products were consistent with the predicted sizes of the derived NS2 proteins, i.e., 40 kDa (NS2ΔN8), 39 kDa (NS2ΔN18), 38 kDa (NS2ΔN25), and 29 kDa (NS2ΔN92). None of the truncated proteins affected the ability of NS2 to form VIBs, as shown by analyses of thin sections of infected cells (data not shown).

To examine whether the truncated proteins bound ssRNA, the unmodified NS2 protein and each of the carboxy- and amino-terminal deleted proteins were precipitated from cell lysates by addition of ammonium sulfate and recovered. The proteins were used in RNA binding studies, and the complexes were analyzed by gel retardation assays as described in Materials and Methods. Complexes were formed with the unmodified NS2 and the two carboxy-terminal deletions but not with any of the four amino-terminal deletions (Fig. 5). The results indicate, therefore, that the amino terminus of NS2 protein is required for RNA binding. In this alternative binding assay, the data confirmed the previous results (Fig. 4) that the carboxy terminus was not required for RNA binding.

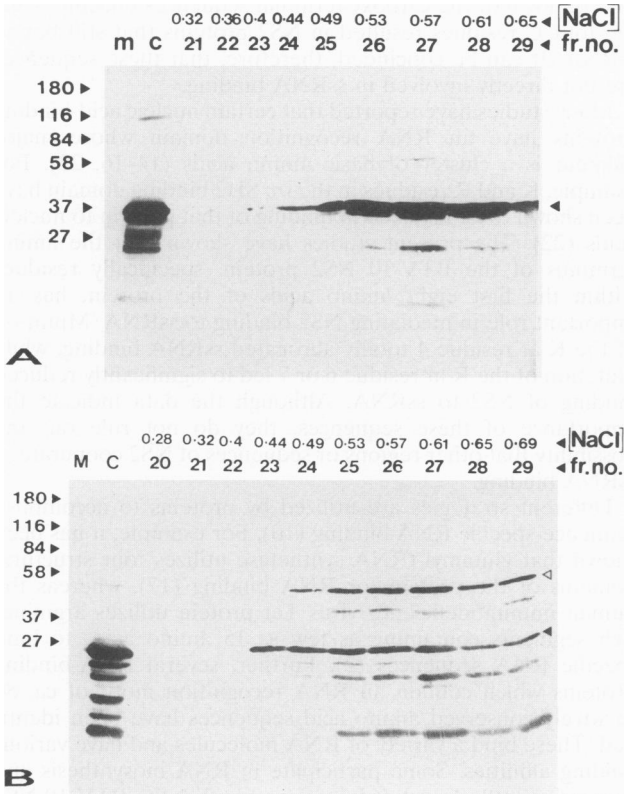

FIG. 4. Poly(U)-Sepharose 4B chromatography of NS2ΔC49 (A) and NS2ΔC130 (B). The respective cell extracts (0.5 ml) were applied to 5-ml poly(U)-Sepharose 4B columns in 0.01 M NaCl in TE buffer and eluted with a gradient of 0.15 to 1.15 M NaCl in TE buffer at a flow rate of 0.2 ml/min. The indicated fractions (fr.no.) corresponding to the NaCl concentrations shown were analyzed by Western blot analyses using anti-BTV-10 rabbit serum after resolution by SDS-PAGE. The sizes (in kilodaltons) and positions of molecular weight markers run in the lanes m (A) and M (B) are indicated. Samples of the original extracts were run in lanes c (A) and C (B).

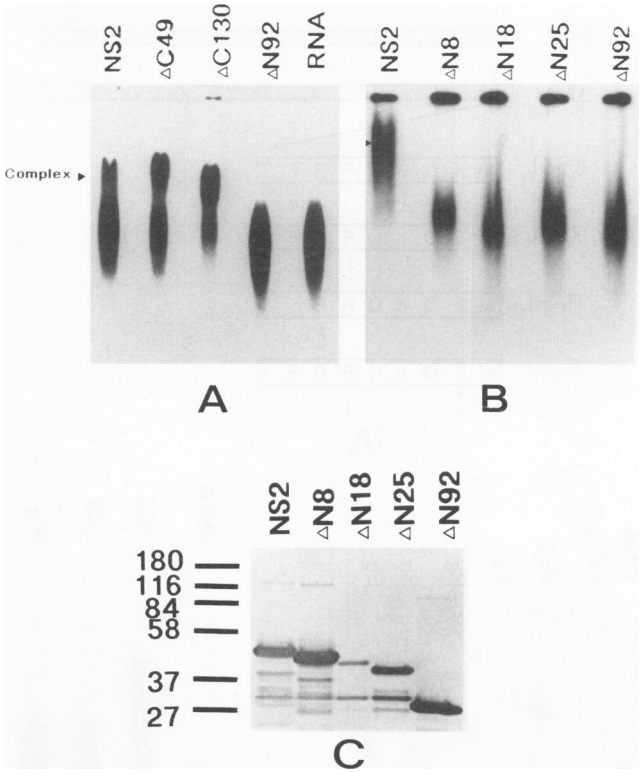

FIG. 5. Gel retardation assays of RNA-NS2 protein complexes. NS2 protein preparations were partially purified as described in Materials and Methods. ^{32}P-labelled ssRNA was prepared and incubated in the presence or absence of 2.5-μg samples of NS2 or the indicated truncated forms of NS2, and the complexes were resolved by electrophoresis on 0.8% agarose gels containing 50 mM glycine, 50 mM Tris (pH 8), and 0.1% sodium deoxycholate. The positions of complexes are indicated (arrowheads). (A) Results obtained with proteins NS2, ΔC49, CΔ130, and ΔN92 as well as an RNA sample incubated in the absence of protein (RNA); (B) results obtained with NS2, ΔN8, ΔN18, ΔN25, and ΔN92; (C) Western blot analyses as described for Fig. 2 to confirm expression of the proteins shown in panel B.

Identification of amino-terminal residues of NS2 associated with RNA binding. In view of the requirement for the amino terminus of NS2 and since it can be predicted that positively charged amino acids in NS2 are probably responsible for nucleic acid binding, the two arginines and the single lysine present at the amino terminus of NS2 were individually mutated to leucines, as illustrated in Fig. 6A. Mutation of the single glutamic acid to leucine was undertaken to serve as a control. As before, the recombinant proteins were partially purified and used in RNA binding studies, and the complexes were analyzed by gel retardation assays (Fig. 6B). Both wild-type NS2 and the glutamic acid mutant (NS2E2L) bound ssRNA and formed complexes. By contrast, mutation of the lysine at amino acid residue 4 (NS2K4L) failed to bind RNA even at high concentrations (Fig. 6C). Mutations of the arginines at residues 6 and 7 (NS2R6L and NS2R7L) gave NS2 proteins that bound much less ssRNA than the controls. These results were further confirmed by poly(U)-Sepharose 4B chromatography (data not shown). In summary, the data indicate the importance of the amino terminus of NS2 for RNA binding and that certain specific amino acids are involved.

DISCUSSION

The affinity of NS2 protein for ssRNA molecules indicates that among other properties, NS2 may have a role in the recruitment of BTV mRNA species for the purposes of RNA encapsidation in maturing virions. Although by using appropriate baculovirus expression vectors it has been shown that all seven structural proteins of BTV can be assembled into core- and virus-like particles in the absence of NS2, in BTV-infected cells, viral proteins and core- and virus-like structures have been identified in association with VIBs (4). It has also been reported that mixtures of soluble NS2 and BTV mRNA form complexes that can be detected by sucrose gradient centrifugation (7). Thus, it is very likely that in some way, NS2 mediates the interaction of the viral mRNA species with core components and nascent cores during the virion assembly process.

Using baculovirus expression systems, we have previously demonstrated that expressed NS2 not only binds ssRNA but accumulates in the cytoplasm as multimers resembling VIBs. In this study, we have generated a number of truncated NS2 proteins and examined their properties in relation to formation of VIBs and ssRNA binding affinity. Deletion at the C termi-

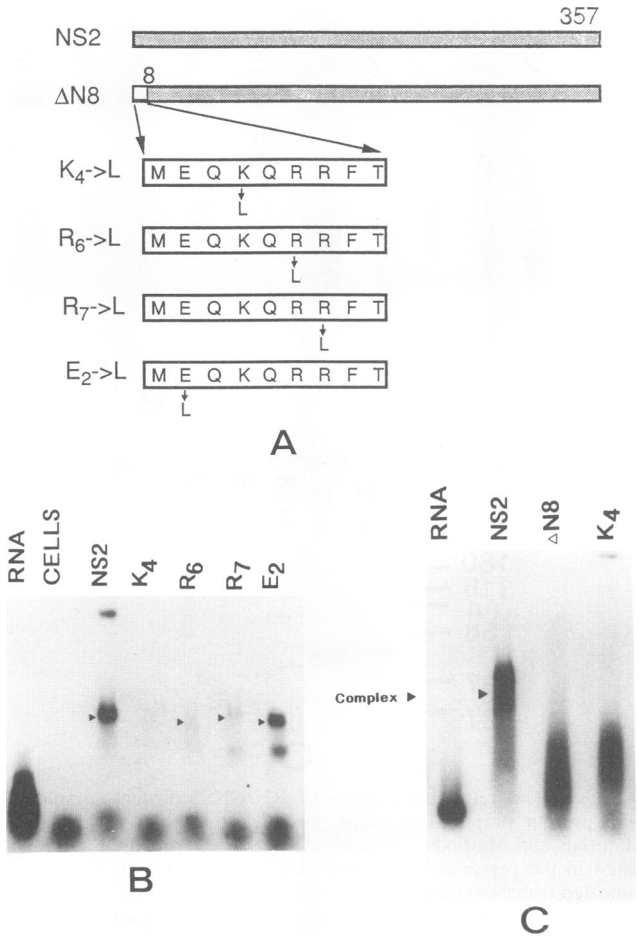

FIG. 6. NS2 point mutations. (A) The top bar is a schematic of the 357-amino-acid NS2 protein. The amino acids at the amino terminus (K4, R6, R7, E2) that were mutated to leucines are shown below. (B) Gel retardation assay with the NS2 point mutants. Lanes 1 (from left to right): ssRNA alone, retardation assay using an extract of uninfected Sf cells, retardation of ssRNA by the unmodified NS2 protein, and retardation assays with the leucine mutants at residues K4, R6, R7, and E2. Positions of the major band of retarded ssRNA are indicated by arrowheads. (C) Gel retardation assay with 10-fold larger quantities of NS2 proteins compared with panel B. Lanes (from left to right): ssRNA, unmodified NS2 protein, NS2ΔN8 mutant protein, and leucine mutant at K4.

nus of up to 130 amino acids of total 357 residues had no effect either on multimerization of the protein or on the ssRNA binding ability. In contrast, deletion of the N terminus or a single mutation at certain residues of the N terminus failed to form any complex with ssRNA, although the mutant retained the ability to form VIBs in the infected cells. The data demonstrated that the two activities of the protein are not associated with each other.

It has been shown that zinc finger motifs occur in many proteins and that they mediate the binding of such proteins to nucleic acids (for a review, see reference 19). The carboxy terminus of the NS2 protein of BTV-10 has the sequence ...PLYCFDESLKRYELQCVGACERVAFVSKDMSLII CRSAFRRL. The cysteines (underlined) do not have an arrangement that is similar to recognized zinc fingers. The three other C residues of NS2 are dispersed in the amino-terminal half of the protein. The studies reported here show that removal of the carboxy-terminal sequences encompassing the four C residues resulted in NS2 proteins that still bound ssRNA. It can be concluded, therefore, that these sequences are not directly involved in ssRNA binding.

Many studies have reported that certain nucleic acid-binding proteins have an RNA recognition domain whose major element is a cluster of basic amino acids (14–16, 22). For example, K and R residues in the *src* SH2 binding domain have been shown to be involved in binding of that protein to nucleic acids (22). The present studies have shown that the amino terminus of the BTV-10 NS2 protein, specifically residues within the first eight amino acids of the protein, has an important role in mediating NS2 binding to ssRNA. Mutation of the K at residue 4 totally abrogated ssRNA binding, while mutation of the R at residue 6 or 7 led to significantly reduced binding of NS2 to ssRNA. Although the data indicate the importance of these sequences, they do not rule out the possibility that other regions or sequences of NS2 cooperate in ssRNA binding.

Different strategies are utilized by proteins to accomplish sequence-specific RNA binding (10). For example, it has been shown that glutamyl-tRNA synthetase utilizes four structural domains of the protein for RNA binding (17), whereas the human immunodeficiency virus Tat protein utilizes arginine-rich segments containing as few as 15 amino acids to bind specific RNA sequences (2). Further, several RNA-binding proteins which contain an RNA recognition motif of ca. 80 relatively conserved amino acid sequences have been identified. These bind a variety of RNA molecules and have various binding affinities. Some participate in RNA biosynthesis and processing (10). As yet, it is unclear whether the BTV-10 NS2 protein is similar to any of these proteins with respect to the ssRNA binding ability and structural motifs. So far, binding activity analyses have shown that NS2 binds ssRNA in a sequence-independent manner (Fig. 4). Similar results have been reported by others (8, 20). What, if anything, determines the sequence specificity of NS2 for BTV ssRNA, or what other components are required to make the binding sequence specific, is not known.

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