Deletion Mapping of the Rotavirus Metalloprotein NS53 (NSP1): the Conserved Cysteine-Rich Region Is Essential for Virus-Specific RNA Binding

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Received 30 December 1993/Accepted 1 March 1994

NS53 (NSP1), the gene 5 product of the group A rotaviruses, is a minor nonstructural protein of 486 to 495 amino acids which binds zinc and contains an amino-terminal highly conserved cysteine-rich region that may form one or two zinc fingers. To study the structure-function of the gene 5 product, wild-type and mutant forms of NS53 were produced by using a recombinant baculovirus expression system and a recombinant vaccinia virus/T7 (vTF7-3) expression system. Analysis of the RNA-binding activity of the wild-type NS53 immobilized onto protein A-Sepharose beads with NS53-specific antiserum showed that the protein exhibited specific affinity for all 11 rotavirus mRNAs. The use of short virus-specific RNA probes indicated that NS53 specifically recognizes an element located near the 5' ends of viral mRNAs. Analysis of the RNA-binding activity of deletion mutants of NS53 showed that the RNA-binding domain resides within the first 81 amino acids of the protein and that the highly conserved cysteine-rich region within this region of the protein is essential for the activity. Gel electrophoresis and Western immunoblot analyses of intracellular fractions derived from infected cells revealed that large amounts of NS53 were present in the cytosol and in association with the cytoskeletal matrix. Indirect immunofluorescence analysis of cells programmed to transiently express mutant forms of NS53 using vTF7-3 indicated that the intracellular localization domain resides between amino acids 84 and 176 of NS53. Together, these data show that the RNA-binding domain and the intracellular localization domain lie upstream from the region of NS53 previously determined not to be essential for replication of rotaviruses in cell culture (J. Hua and J. T. Patton, *Virology* 198:567–576, 1994).

Rotaviruses, members of the family *Reoviridae*, are the major etiological agents of acute gastroenteritis in infants and young children (3). The rotavirus virion consists of 3 concentric layers of protein and 11 segments of double-stranded RNA (41, 51). The genome is replicated asymmetrically, with viral mRNA serving as the template for the synthesis of minus-stranded RNA, producing double-stranded RNA (35, 36). In addition to the six proteins that make up the virion, the viral genome encodes five or six nonstructural proteins (13). Although their precise functions in replication remain undefined, the nonstructural proteins are essential for the formation of infectious virions. Three of the nonstructural proteins, NS53 (NSP1; 58 kDa) (6), NS35 (NSP2; 37 kDa) (23), and NS34 (NSP3; 35 kDa) (30, 40), possess RNA-binding activity and are components of rotavirus replication intermediates (16, 37). Previous studies indicate that NS35, a highly conserved basic protein (38), has nonspecific RNA-binding activity (23), accumulates in cytoplasmic inclusions, i.e., viroplasms, of infected cells (39), forms multimers, and interacts with the viral RNA polymerase, VP1 (125 kDa) (24). Characterization of the rotavirus temperature-sensitive mutant, *tsE* (17), indicates that NS35 also participates in viral RNA packaging (42) and is necessary for genome replication (9). In comparison, NS34 is a slightly acidic protein that is associated with the cytoskeleton of infected cells and is capable of oligomerization (30). Pro-

tein-RNA cross-linking studies indicate that NS34 preferentially binds to the 3' termini of viral mRNAs (40).

Of the nonstructural proteins, the gene 5 product NS53 is the least abundant in the infected cell (12). NS53 is also unique among the nonstructural proteins of the group A rotaviruses in that it is variable in length, e.g., strain Wa contains 486 amino acids and strain SA11 contains 495 amino acids, and poorly conserved, especially within the COOH half of the protein (19, 32). Near the amino terminus, NS53 contains the cysteine-rich sequence C-X₂-C-X₈-C-X₂-C-X₃-H-X-C-X₂-C-X₅-C which has been detected in the gene 5 products of all group A and C rotaviruses examined to date (4, 19). The cysteine-rich motif has been proposed to form one or two zinc fingers and, by analogy with other proteins that contain such structures (1, 2, 14), may be important for the RNA-binding activity of NS53 (4, 19, 32). Consistent with the hypothesis that NS53 contains a metal-binding domain is the observation that the protein has affinity for zinc (6). Examination of rotavirus-infected cells by indirect immunofluorescence (IF) assay and subcellular fractionation has provided evidence that NS53 accumulates in the cytoplasm, possibly in association with the cytoskeleton (20). IF assay indicates that viroplasms do not contain NS53.

Characterization of brvA (21) has shown that this bovine rotavirus variant contains a rearranged gene 5 which encodes a carboxyl-truncated NS53 of 258 amino acids (20). In infected cells, distribution of the truncated protein is like that of wild-type NS53 (491 amino acids), suggesting that the intracellular localization domain resides in the amino-terminal half of the protein. Given that brvA and P9Δ5, a bovine variant proposed to encode a carboxyl-truncated NS53 of only 150 amino acids (47), are nondefective with respect to growth in cell culture, it is apparent that the carboxyl-terminal half of

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NS53 is not necessary for virus replication. However, it remains possible that the carboxyl portion of the protein is essential *in vivo*, perhaps influencing the host range of the virus or serving as a virulence determinant (5, 20).

To gain additional insight into the structure and function of NS53, we mapped by deletion mutagenesis the location of functional domains in the protein important for RNA binding and intracellular localization. The RNA-binding domain was mapped to the first 81 amino acids of NS53, a region that includes the putative zinc finger(s) of the protein. Characterization of the binding activity indicated that it was specific for the 5' ends of rotavirus mRNAs. The intracellular localization domain was mapped to a region in NS53 of approximately 100 amino acids located immediately downstream from the RNA-binding domain. No function was identified for the carboxyl-terminal 300 amino acids of NS53, which include the region of the protein shown previously not to be required for virus replication.

MATERIALS AND METHODS

Cells and virus. MA104 and CV-1 cells were grown in Eagle's minimal essential medium containing 10% fetal bovine serum. Simian rotavirus SA11 was propagated in MA104 cells, and virions were purified from infected cell lysates by centrifugation on CsCl gradients (37). The recombinant vaccinia virus vTF7-3 which expresses T7 RNA polymerase was provided by B. Moss (National Institutes of Health) (15). vTF7-3 was propagated in CV-1 cells, and the virus titer was determined by plaque assay on monolayers of the same cells. *Spodoptera frugiperda* (Sf9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from Invitrogen Corporation. Sf9 cells were grown in TNM-FH medium (45) containing 10% fetal bovine serum. Wild-type and recombinant baculoviruses were propagated on Sf9 cell monolayers, and virus titers were determined by plaque assay (45).

Production of NS53-specific antiserum. The oligopeptide, NH₂-CGTLTEEFELLISNSEDNE-COOH, which includes the last 19 amino acids of the SA11 NS53 protein, was obtained from the Protein Chemistry Core Laboratory at the University of Florida. The peptide was conjugated to the carrier protein, keyhole limpet hemocyanin, according to the instructions of the supplier (Pierce Chemical Co.). Hyperimmune antiserum was generated by intradermal injection of 200 µg of the conjugate emulsified in complete Freund's adjuvant at multiple sites along the back of a New Zealand White rabbit (50). The animal was given booster injections with the conjugate emulsified in incomplete Freund's adjuvant at 14, 28, 35, and 42 days after primary immunization. The SA11 NS53-specific serum, termed C19 antiserum, was collected 1 week after the last injection.

Construction of recombinant baculoviruses. An SA11 gene 5 cDNA containing 5'- and 3'-terminal *NheI* sites was generated by PCR as described previously, with the following cycling conditions: 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C for 30 cycles (11). The reaction mixture included as template, pUC18g5, which contains a full-length SA11 gene 5 cDNA (32), the forward primer, 5'-CTGGTGGCTAGCATGGCTACTTTAAAGATGC-3', and the reverse primer, 5'-TCCCGTGCTAGCGCGCTACTCTAGTCTGG-3'. The *NheI* sites are underlined. The PCR product was gel purified and ligated into the vector pCR1000 (Invitrogen Corporation). The resulting construct, pCRg5N, was digested with *NheI*, and the gene 5-specific fragment (g5-*NheI*) was ligated into the baculovirus transfer vector pBlueBac (Invitrogen Corporation) which had been cleaved with *NheI*. Constructs of pBlueBac

FIG. 1. Deletion mutagenesis of NS53. (A) Diagram of a partial restriction map of the transcription vector, pSP72g5, that contains gene 5 cDNA positioned downstream from the promoter (prm) for T7 RNA polymerase. Numbers correspond to nucleotide positions in full-length gene 5. Locations in the cDNA for which oligonucleotide primers were prepared are indicated as P1 to P4. (B) Schematic of the mutant NS53 species used in this study. Numbers correspond to amino acid (aa) positions in NS53. The locations of the conserved basic region, cysteine-rich region (CRR), and the C19-specific (Ab) epitope are indicated. (C) Sequences and nucleotide positions of oligonucleotide primers used to delete the CRR of NS53.

that contained the gene 5 insert (pBlueBac-g5) were identified on the basis of antibiotic resistance and restriction enzyme analysis (28). Recombinant baculoviruses containing gene 5 were generated by cotransfection of the transfer vector and wild-type baculovirus DNA into Sf9 cells using the calcium phosphate precipitation technique (45). Recombinant baculoviruses containing gene 5 (vBlueBac-g5) were identified on the basis of their ability to produce blue plaques on Sf9 cell monolayers in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and to direct the synthesis of high levels of NS53 (49).

Deletion mutagenesis of NS53. A restriction map of the gene 5 cDNA relevant to the mutagenesis of NS53 is shown in Fig. 1. To produce a T7 transcription vector encoding full-length NS53 (pSP72g5), the fragment g5-*NheI* was blunt-ended with mung bean nuclease and ligated into the plasmid pSP72 which had been digested with *HindIII* and *EcoRV* and blunt-ended with mung bean nuclease (New England BioLabs). To produce the transcription vector pSP72g5d9-83 (NS53d9-83), which encodes a mutant form of NS53 that lacks amino acids 9 to 83 of the open reading frame, pSP72g5 was digested with *SphI* and then treated with DNA polymerase (Klenow fragment) to

fill in the 3'-recessed ends. Afterwards, the DNA was digested with *Hind*III and treated with mung bean nuclease. The resulting 2.8-kb fragment was recircularized by self-ligation. To generate the transcription vector pSP72g5d177-359 (NS53 d177-359), pSP72g5 was digested with *Hpa*I and the 2.9- and 0.6-kb DNA fragments were gel purified and the 2.9 kb fragment was treated with alkaline phosphatase. The 0.6-kb fragment was then ligated into the 2.9-kb fragment. The vector pSP72g5d82-476 (NS53d82-476) was made by digesting pSP72g5 with *Eco*RI and *Hind*III and then gel purifying the 2.8-kb DNA fragment. After treatment with mung bean nuclease, the 2.8-kb fragment was recircularized by self-ligation. To produce the transcription vector pSP72g5d177-476 (NS53 d177-476), pSP72g5d177-359 was digested with *Hpa*I and *Eco*RI and the 2.5- and 0.6-kb DNA fragments were gel purified. The 0.6-kb fragment was ligated into the 2.5-kb fragment (pSP72HE) which had been treated with mung bean nuclease and alkaline phosphatase.

PCR was used to generate pSP72g5dCRR (NS53dCRR), a transcription vector that encodes a form of NS53 that lacks the conserved cysteine-rich region (amino acids 41 to 81). The nature of the four primers used in the amplification reactions is shown in Fig. 1C. To produce pSP72g5dCRR, two amplification reactions were initially performed, with both containing as template pSP72g5, but one reaction contained primers 1 and 3 and the other contained primers 2 and 4. The amplified DNAs of both reactions were then combined and added to a second amplification reaction mixture containing primers 1 and 4. The PCR product was then ligated into pSP72HE. Dideoxynucleotide sequencing was used to verify sequences of plasmids encoding NS53 deletion mutants (43).

Subcellular fractionation of rotavirus-infected cells. Monolayers of MA104 cells were infected with 10 PFU of trypsin-activated SA11-4F rotavirus per cell and maintained in 80% Met-Cys-free Eagle's minimal essential medium containing 10 μ Ci of 35 S-amino acids (>1,000 Ci/mmol) per ml beginning at 4 h postinfection. At 9 h postinfection, the cells were lysed and separated into soluble, cytoskeletal, and nuclear fractions on the basis of previously reported procedures (7, 8, 20). Cells were washed with phosphate-buffered saline, gently suspended in cold lysis buffer [10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 0.3 M sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride], and incubated on ice for 3 min. The nuclei and attached cytoskeleton were separated from the soluble fraction by low-speed centrifugation. The cytoskeleton was sheared from the nuclei by resuspending the pellet in cold CK extraction buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1% Tween 20, 0.5% deoxycholate) and extensive Dounce homogenization. The nuclear fraction was separated from the cytoskeletal fraction by low-speed centrifugation.

Expression of wild-type and mutagenized NS53. To express wild-type and mutagenized forms of NS53 in CV-1 cells, T7 transcription vectors bearing intact or altered gene 5 coding sequences were transfected into CV-1 cells infected with 5 to 10 PFU of vTF7-3 per cell (20). To radiolabel the expressed proteins, the transfected cells were maintained in 90% Met-Cys-free Eagle's minimal essential medium containing 20 μ Ci of 35 S-amino acids (>1,000 Ci/mmol) per ml. At 15 h postinfection, the cells were scraped into isotonic buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and pelleted by low-speed centrifugation. The cells were then lysed by suspending them into buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.6, 150 mM NaCl, 0.5 mM disodium EDTA, 1 mM phenylmethylsulfonyl fluoride,

and 1% Triton X-100. The nuclear and cytoskeletal fractions were recovered from cell lysates by low-speed centrifugation. To release NS53 from the cytoskeletal-nuclear fraction into the soluble fraction, lysates were subjected to extensive Dounce homogenization prior to low-speed centrifugation.

Baculovirus recombinant NS53 was made by infecting Sf9 cells with 10 PFU of vBlueBac-g5 per cell and then maintaining the cells in Grace's medium until harvest at 72 h postinfection (45). To label the expressed NS53, the Sf9 cells were placed in methionine-free Grace's medium containing 20 μ Ci per ml of 35 S-amino acids (>1,000 Ci/mmol). Cells were harvested and fractionated as described above for the vTF7-3-infected CV-1 cells.

Synthesis of RNA probes. To produce a transcription vector that in the presence of T7 RNA polymerase would synthesize gene 5 mRNA containing authentic 5' and 3' termini, a cDNA of gene 5 was generated by PCR that was full-length except that it lacked the 5'-terminal dinucleotide GG. The amplification reaction included as template pSP72g5, the forward primer 5'-CTTTTTTTTGAAAAGTCT-3', and the reverse primer 5'-GGTTCACAGTATTTTGCC-3'. The gene 5 PCR product was ligated into the T7 transcription vector v2.0 that had been digested with *Stu*I and *Sma*I and treated with alkaline phosphatase (34). Because the vector contains two G residues between the T7 promoter and the *Stu*I-*Sma*I insertion site, introduction of the PCR product into the vector regenerated the 5'-terminal dinucleotide GG found in the authentic gene 5 RNA. Immediately downstream from the virus-specific sequence of v2.0g5 is positioned a hepatitis D virus ribozyme that autocatalytically cleaves transcripts synthesized from the vector following the 3'-terminal C residue found in gene 5 RNA (34). Downstream from the ribozyme is a T7 transcriptional terminator.

To generate an RNA probe representing the 3' end of gene 5, a DNA fragment extending between nucleotide 1042 and the 3' end (nucleotide 1611) of gene 5 was synthesized by PCR. The amplification reaction included as template v2.0g5, the forward primer 5'-TTGGCGGATTCGTG-3', and the reverse primer 5'-TCCGCGGTTACAGTATTTT-3' which also contained the recognition sequence for *Sac*II (underlined). The PCR product was ligated into the vector v2.0 and digested with *Stu*I and *Sma*I, producing v2.0g5-3'.

32 P-labeled viral mRNA was synthesized in reaction mixtures containing activated SA11 single-shelled particles (10). Full-length 32 P-labeled gene 5 mRNA was synthesized in vitro in reaction mixtures containing v2.0g5 and T7 RNA polymerase. The 32 P-labeled 5'- or 3'-specific gene 5 probes, s5-5' and s5-3' RNA, were synthesized by runoff transcription in reaction mixtures containing T7 RNA polymerase and *Hind*III-linearized v2.0g5 and *Sac*II-linearized v2.0g5-3', respectively (31). The RNA probes were radiolabeled by including 2 mCi per ml of [32 P]UTP (650 Ci/mmol) in RNA synthesis reactions. The 5' end of s5-5' RNA (278 nucleotides) and the 3' end of s5-3' RNA (570 nucleotides) are authentic with respect to gene 5 RNA.

Immunoprecipitation, electrophoresis, and Western immunoblot analysis. For immunoprecipitation, soluble fractions (250 to 300 μ l) derived from cell lysates after Dounce homogenization were brought to a final volume of 1 ml with buffer A (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.5 mM disodium EDTA, 100 μ M ZnCl₂, 5% glycerol, 5 mM β -mercaptoethanol, and 0.1% Triton X-100). After the addition of NS53 C19 antiserum to a final dilution of 1:1,000, the samples were incubated overnight at 4°C with mixing and then incubated for 2 h with protein A-Sepharose beads. Afterwards, the beads

were washed three times with buffer A. Protein was eluted from the beads by boiling them in Laemmli sample buffer (23).

Proteins in samples obtained from MA104, CV-1, and Sf9 cells and in immunoprecipitates were analyzed by electrophoresis on 12 or 20% polyacrylamide gels containing sodium dodecyl sulfate (26). ^{35}S -labeled proteins were detected in the gels by fluorography (27). Detection of NS53 in cellular fractions by Western blot analysis using C19 antiserum was performed according to published procedures (48).

RNA-binding analysis. Soluble fractions from CV-1 and Sf9 cell lysates containing NS53 were diluted into buffer A and incubated with C19 antiserum, and the antibody-antigen complexes were adsorbed onto protein A-Sepharose beads according to the procedures described above for immunoprecipitation. The immunoadsorbed NS53 was then used as an NS53-specific affinity matrix in RNA-binding assays (25). Each reaction mixture contained approximately 25 μl of NS53-coated beads and 2×10^5 to 5×10^5 cpm of ^{32}P -labeled RNA probe in 0.6 ml of buffer A. As nonspecific competitors, yeast tRNA and brome mosaic virus (BMV) mRNA (Promega) were added to some reaction mixtures. After incubation at room temperature for 30 min, the beads were washed four times with binding buffer. Radioactivity bound to the beads was assayed by using a Beckman scintillation counter. To recover the bound ^{32}P -RNA, the beads were suspended in binding buffer containing 1 M NaCl. After centrifugation to remove the beads, RNA in supernatants was analyzed by electrophoresis on 6% polyacrylamide gels containing 7 M urea and by autoradiography (29).

Indirect IF assay. Nearly confluent monolayers of MA104 cells grown on chamber slides were mock infected or infected with 5 to 10 PFU of SA11 rotavirus or vTF7-3 per cell and afterwards transfected with T7 transcription vectors using calcium phosphate (20). At 8 to 12 h postinfection, the cells were processed for IF assay by using a 1:600 dilution of C19 antiserum and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibody as described previously (20).

RESULTS

Baculovirus-expressed NS53 binds viral mRNA. A recombinant baculovirus, vBlueBac-g5, was generated that contained a cDNA of the open reading frame for SA11 gene 5 located under the control of the promoter for the polyhedron gene of the wild-type baculovirus, AcNPV. Pulse-labeling experiments showed that a protein of the size of NS53 (58 kDa) was expressed in vBlueBac-g5-infected cells, with maximal levels of synthesis occurring at 48 h postinfection (Fig. 2) and maximal levels of accumulation achieved at 72 h postinfection (data not shown). The 58-kDa protein was not detected in AcNPV-infected cells. To verify that the 58-kDa protein represented the product of SA11 gene 5, an NS53-specific antiserum, C19, was prepared by immunizing a rabbit with a peptide identical in sequence to the carboxyl-terminal 19 amino acids of NS53. Assay of lysates from vBlueBac-g5 and AcNPV-infected Sf9 cells by immunoprecipitation using preimmune serum and C19 antiserum showed that the 58-kDa protein produced in cells infected by the recombinant baculovirus was the gene 5 product, NS53 (Fig. 2).

To examine the ability of the recombinant NS53 to bind RNA, lysates from vBlueBac-g5-infected cells were incubated with C19 antiserum and then the antibody-antigen complex was adsorbed onto protein A-coated Sepharose beads. Afterwards, the NS53-coated beads were assayed for the RNA-binding activity by incubation with ^{32}P -labeled viral mRNA made in vitro by transcriptionally active SA11 virions. In

FIG. 2. Expression of recombinant NS53. Sf9 cells were infected with the recombinant baculovirus vBlueBac-g5 (r) or with wild-type baculovirus (wt). After pulse-labeling for 2 h at 24, 48, and 72 h postinfection with ^{35}S -amino acids, cytoskeleton-containing fractions of the infected cells were prepared and analyzed by electrophoresis on a 12% polyacrylamide gel. The soluble fraction recovered at 48 h postinfection from Sf9 cells infected with vBlueBac-g5 or wildtype baculovirus were subjected to immunoprecipitation (Imppt) using NS53-specific C19 antiserum (NS53 Ab) or preimmune serum (PI). Closed and open arrowheads indicate NS53 and the polyhedron protein, respectively.

contrast to control beads that were prepared in the absence of either the NS53 lysate or the C19 antiserum, the NS53-coated beads bound high levels of viral mRNA (Fig. 3A). Electrophoretic analysis of the mRNA which was bound to the beads showed that NS53 had affinity for all 11 species of rotavirus mRNAs (Fig. 3B). NS53 exhibited no obvious preferential affinity for any of the viral mRNAs. The addition of increasing concentrations (10 to 150 ng) of cold viral mRNA to the binding reaction mixtures successfully decreased the levels of ^{32}P -labeled viral mRNA that associated with the NS53-coated beads. In contrast, the level of viral mRNA bound by the NS53-coated beads was not affected by the presence of high concentrations (15 μg) of yeast tRNA in the binding reaction mixture, suggesting that NS53 is a virus-specific RNA-binding protein.

RNA-binding domain of NS53 includes the zinc finger motif. To locate the RNA-binding domain within NS53, a cDNA of SA11 gene 5 was placed into a transcription vector under the control of the promoter for T7 RNA polymerase (pSP72g5; Fig. 1). Using appropriate restriction enzymes and PCR-based procedures, deletions of various sizes and locations were then introduced into the open reading frame for gene 5 in the vector pSP72g5 (Fig. 1). All the deletion vectors were constructed such that they retained the carboxyl 19 amino acids of the authentic NS53 protein and, hence, also retained the epitope recognized by the C19 antiserum. The full-length and mutant species of NS53 were expressed by transfection of transcription vectors into CV-1 cells infected with vTF7-3, a recombinant vaccinia virus which constitutively synthesizes T7 RNA polymerase. Electrophoretic analyses of the cytoskeleton-containing fractions of these cells showed that they produced novel proteins of the sizes predicted for the wild-type and truncated species of NS53 (Fig. 4A). The novel proteins were deemed to

FIG. 3. Affinity of baculovirus recombinant NS53 for viral mRNA. Extracts prepared from vBlueBac-g5- and wild-type baculovirus-infected Sf9 cells were incubated with NS53-specific antiserum and protein A-Sepharose beads. (A) The beads were incubated with 2×10^5 cpm (3 ng) of ³²P-labeled viral mRNA, washed, and assayed for radioactivity. As competitor RNA, a molar excess (X) of unlabeled viral mRNA and tRNA were included in some reactions. The column height and error bar represent the average radioactivity bound and range, respectively, for four separate experiments. (B) ³²P-labeled mRNA bound to the protein A beads was analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Lane numbers in panel B correspond to those of panel A.

be of gene 5 origin as they were recognized by the C19 antiserum in immunoprecipitation assays (Fig. 4B).

The wild-type and mutant species of NS53 expressed in CV-1 cells were allowed to form complexes with the C19 antibodies which were then adsorbed onto protein A-Sepharose beads. The NS53-coated beads were incubated with ³²P-labeled viral mRNA, and the amount of bound RNA was determined. As found for NS53 expressed in vBlueBac-g5-infected Sf9 cells, wild-type NS53 expressed in CV-1 cells possessed an affinity for radiolabeled viral mRNA that could be inhibited by using unlabeled viral mRNA (Fig. 5A). The

FIG. 4. Expression of wild-type and mutagenized species of NS53. vTF7-3-infected CV-1 cells were mock transfected or transfected with plasmids encoding wild-type or mutant forms of NS53 and maintained in ³⁵S-amino acids. (Upper panel) Cytoskeleton-containing fractions of the cells were analyzed by polyacrylamide gel electrophoresis. (Lower panel) Soluble fractions obtained after Dounce homogenization of the cells were subjected to immunoprecipitation with NS53-specific antiserum. Cells were mock transfected or transfected with pSP72g5 (wt), pSP72dCRR, pSP72g5d9-83, pSP72g5d177-359, pSP72g5d177-476, and pSP72g5d82-476, respectively. The expected NS53-specific products are indicated with closed arrowheads. A truncated NS53-specific product (open arrowheads) was detected in cells after transfection with pSP72g5dCRR, probably because of some protein synthesis initiating at Met-31.

binding of mRNA was dependent on the presence of NS53 on the beads. The extent of binding of viral mRNA to NS53-coated beads was not affected by the presence of a 250-fold excess of BMV mRNA over the radiolabeled probe, providing additional evidence that NS53 possessed virus-specific RNA-binding activity (Fig. 5A).

Like wild-type NS53, mutant forms of NS53 that contained the first 81 amino acids of the protein, i.e., NS53d177-359 and NS53d82-476, and therefore included the conserved cysteine-rich region (residues 42 to 75) retained the capacity to bind full-length ³²P-labeled gene 5 mRNA (Fig. 5B). In contrast, mutant forms of NS53 that lacked the cysteine-rich region, i.e.,

FIG. 5. Mapping of the RNA-binding domain of NS53. Soluble extracts obtained after Dounce homogenization of cells either mock transfected or transfected with plasmids encoding wild-type or mutant forms of NS53 were incubated sequentially with NS53-specific antiserum and protein A-Sepharose beads. After being washed, the NS53-coated beads were incubated with ³²P-labeled RNA probe. (A) Reaction mixtures included a probe 2×10^5 cpm of ³²P-labeled viral mRNA, protein A beads coated with wild-type NS53, and in some cases, a molar excess (X) of cold viral mRNA and BMV mRNA. (B) Reaction mixtures included 5×10^5 cpm of ³²P-labeled gene 5 mRNA made from the transcription vector v2.0g5 and protein A beads coated with wild-type or mutant NS53 (open bars) or mock-coated protein A beads (hatched bars). The column height and error bar represent the average radioactivity bound and range, respectively, for three separate experiments.

NS53dCRR and NS53d9-83, were unable to bind the mRNA. When considered together, the results with deletion mutants indicate that the RNA-binding domain of NS53 resides within the first 81 amino acids of the protein and that the cysteine-rich region within this portion of the protein is essential for RNA-binding activity.

RNA-binding domain of NS53 specifically recognizes 5'-termini of viral mRNAs. The specificity of the RNA-binding activity of NS53 was assessed by using the ³²P-labeled RNA probes s5-5' and s5-3', which were synthesized in vitro by runoff transcription. The probes were identical in sequence to

FIG. 6. Specificity of NS53 RNA-binding activity. NS53 was expressed in vTF7-3-infected cells transfected with pSP72g5 and used to prepare NS53-coated protein A-Sepharose beads. The beads were incubated in reaction mixtures containing 5×10^5 cpm (1 ng) of the ³²P-labeled 5'-specific probe s5-5' (A) or 2×10^5 cpm (1 ng) of the 3'-specific probe s5-3' (B). As shown below each panel, some reaction mixtures also contained a molar excess (X) of cold probe or BMV mRNA. The column height and error bar represent the average radioactivity bound and range, respectively, for three separate experiments.

the first 278 nucleotides (s5-5') and to the last 570 nucleotides (s5-3') of the gene 5 mRNA. The probes were incubated with NS53-coated Sepharose beads, and the amount of bound probe was measured. As shown in Fig. 6A, significant levels of ³²P-labeled s5-5' RNA were bound by NS53. The binding of the labeled 5'-specific probe to NS53 was nearly completely blocked by the addition of excess cold probe to binding reaction mixtures but not by the addition of excess BMV mRNA. NS53 also bound the ³²P-labeled s5-3' RNA, but the association of the 3' probe with NS53 was not resistant to competition by BMV mRNA (Fig. 6B). Together, these results indicate that NS53 has specific binding affinity for a sequence or structure at the 5' end of rotavirus mRNAs but, as reported previously for the protein (6), also possesses nonspecific RNA-binding activity.

Detection of NS53 in the soluble and cytoskeleton fractions

FIG. 7. Intracellular location of NS53. The cytoskeletal fraction of vTF7-3-infected CV-1 cells, either mock transfected (lane 1) or transfected with pSP72g5 (lane 2), and the soluble (lanes 3 and 4) and cytoskeletal (lanes 5 and 6) fractions and nuclei (lanes 7 and 8) of MA104 cells, either mock infected (lanes 3, 5, and 7) or infected with SA11 rotavirus (lanes 4, 6, and 8), were analyzed by polyacrylamide gel electrophoresis (A). NS53 in the nuclei and the cytoskeletal and soluble fractions of SA11-infected cells (lanes 9, 10, and 11, respectively) was detected by Western blot assay using C19 NS53-specific antiserum (B).

of infected cells. IF staining has suggested that NS53 is associated with the cytoskeletal fraction of bovine rotavirus-infected cells (20). Additional information concerning the intracellular location of NS53 was obtained by using detergent-based procedures to prepare soluble, nuclear, and cytoskeletal fractions of SA11-infected cells. The protein content of the fractions was analyzed by gel electrophoresis, and the location of NS53 was identified by Western blot assay (Fig. 7). Densitometric analysis showed that approximately 90% of the gene 11 product, NS26, was located in the soluble portion of the cell. In contrast, most NS34 (70%) existed in association with the cytoskeletal fraction, a characteristic expected on the basis of previous studies involving this protein (30). Although significant levels of NS35 were detected in both the soluble and cytoskeletal fractions of the cell, most (70% of the total) was detected in the former fraction than in the latter. By direct inspection of gels analyzing the protein composition of the cellular fractions, it was difficult to identify the NS53-specific band, which was not unexpected given the low levels of this protein in rotavirus-infected cells. By Western blot analysis, high levels of NS53 were detected in both the soluble and cytoskeletal fractions of the cell (Fig. 7B), although more NS53 generally copurified with the cytoskeletal fraction than was recovered in the soluble fraction. When the intracellular distribution of NS53 is contrasted with that of the soluble component NS26 and the cytoskeleton component NS34, NS53 would appear to possess affinity for the cytoskeletal matrix of the infected cell, albeit possibly transient or weak in nature. Indirect IF assay of SA11-infected cells using the C19 antiserum confirmed that NS53 was present in the cytoplasm but was not associated with viroplasms (Fig. 8). In the cytoplasm, the distribution of NS53 was slightly punctate and filamentous, a pattern consistent with results from the cellular fractionation studies, indicating that some of the protein accumulated on the cytoskeleton. The distribution pattern of NS53 in the infected cell is clearly different from that of NS35, an RNA-binding protein which accumulates in viroplasms (Fig. 8).

When NS53 was expressed in vTF7-3-infected cells by transfection of the plasmid pSP72g5, the distribution of NS53 as judged by IF staining was indistinguishable from that found in SA11-infected cells (Fig. 8). Hence, the pattern of NS53

intracellular distribution of NS53 in the infected cell is probably not dependent on the presence of any other viral protein.

Location of the intracellular localization domain of NS53. Previous studies on the bovine rotavirus variant brvA indicated that the intracellular localization domain of NS53 resides in the first 258 amino acids of the protein (20). To identify the position of the localization domain, transcription vectors encoding mutant species of NS53 were transfected into CV-1 cells infected with vTF7-3. The distribution of the NS53 species in the cells was then determined by indirect IF assay using the C19 antiserum (Fig. 8). The results showed that mutant species of NS53 lacking the conserved cysteine-rich region and sequences upstream of it (NS53d9-83) localized in a manner indistinguishable from that found for full-length NS53 expressed in cells by transfection with pSP72g5 or by infection with SA11 rotavirus (Fig. 8). Likewise, an NS53 mutant lacking amino acids 177 to 476 (NS53d177-476) showed a fluorescence staining pattern similar to that of full-length NS53. In contrast, the distribution of mutant NS53 lacking amino acids 82 to 476 (NS53d82-476) differed markedly from that obtained for full-length NS53. In the case of NS53d82-476, the NS53 species accumulated primarily in association with the nucleus, indicating that the intracellular localization domain of wild-type NS53 that normally leads to the association of some of the protein with the cytoskeleton was missing or altered in this deletion mutant (Fig. 8). When considered together, these data indicate that the intracellular localization domain resides between amino acids 84 and 176 of NS53.

DISCUSSION

Although NS53 exhibits poor overall amino acid homology among the group A rotaviruses, conserved near the amino terminus of the protein are three basic regions and the cysteine-rich motif C-X₂-C-X₈-C-X₂-C-X₃-H-X-C-X₂-C-X₅-C (Fig. 9). By analogy with other metalloproteins, the cysteine-rich motif has been proposed to form one or two zinc fingers (4, 19, 32). Functional analyses of zinc fingers have shown that such structures generally impart specific nucleic acid-binding activities to proteins (1, 2). In this study, we have shown that NS53 produced by a recombinant baculovirus and by a vTF7-3

FIG. 8. IF staining of cells expressing wild-type and mutant NS53. MA104 cells were infected with SA11 rotavirus and fixed at 8 h postinfection, and CV-1 cells were infected with vTF7-3 and transfected with pSP72g5 (NS53wt), pSP72g5d9-83 (NS53d9-83), pSP72g5d177-359 (NS53d177-359), pSP72g5d177-476 (NS53d177-476), and pSP72g5d82-476 (NS53d82-476) or they were mock transfected and fixed at 14 h postinfection. The cells were incubated with C19 NS53-specific antiserum or anti-NS35 monoclonal antibody 952/122 and then with fluorescein isothiocyanate-conjugated anti-rabbit antibody. The staining patterns of NS53 and NS35 were examined for fluorescence and photographed on an Olympus BHTU IF microscope at 400 $\times$ magnification.

FIG. 9. Structure and function of NS53. The locations of the conserved basic regions and cysteine-rich regions of NS53 are derived from comparative sequence information that include group A and C rotaviruses (19). The region of 150 to 258 amino acids of NS53 is proposed to be nonessential for growth of rotavirus in cell culture on the basis of the predicted RNA sequence for gene 5 of the variant P9Δ5 (47). The nondefective variant brvA has been shown to produce in infected cells a truncated NS53 that lacks amino acids beyond residue 258 (20).

transient expression system has specific affinity for rotavirus mRNAs. To locate the RNA-binding domain in NS53, we prepared a set of gene 5 deletion mutants lacking various regions of the open reading frame of NS53 and examined these mutants for the ability to bind rotavirus mRNA. When the cysteine-rich region of NS53 was deleted, the protein lost the ability to bind RNA. This finding taken with the results of earlier experiments showing that NS53 is a zinc-binding protein provides compelling evidence that the cysteine-rich region forms zinc fingers that function in RNA binding (6).

Our experiments have indicated that the RNA-binding activity of NS53 is specific, because it binds rotavirus mRNAs in a manner that cannot be reversed by the presence of competitor nonviral RNA. The use of virus-specific RNA probes in binding reactions suggests that the recognition signal bound by NS53 is located within the first 278 nucleotides of rotavirus mRNAs. While more experiments are required to identify the nature of the signal, our data provide evidence that it is present in all 11 rotavirus mRNAs. The signal may be the 5'-terminal sequence, 5'-G₂-C-T-T/A-T-A/T₄-3', because it is the only sequence that is remarkably conserved in the first 278 nucleotides of rotavirus mRNAs. However, because studies of the *Xenopus* transcription factor TFIIA indicate that it is the secondary and tertiary structures of RNA, and not its primary sequence, that are recognized by RNA-binding proteins containing zinc fingers (46), it may be the putative secondary structures at the 5' ends of rotavirus mRNAs which serve as the recognition signals for NS53 (19, 38).

Electrophoretic analyses of fractions obtained from rotavirus-infected cells indicated that NS53 accumulates in the cytoplasm, with large amounts present both in the cytosol and associated with the cytoskeletal matrix. The somewhat filamentous distribution of NS53 in SA11-infected cells as judged by IF staining (Fig. 8) provided additional evidence of the possible interaction of NS53 with the cytoskeletal network of infected cells. In experiments described in this report, we used IF staining to analyze the distribution pattern of NS53 deletion mutants that were transiently expressed in vTF7-3-infected CV-1 cells. By this assay, the distribution of NS53 in the cell was found to be affected only when amino acids 84 to 176 of the protein were deleted, thus defining an essential region necessary for the correct intracellular localization of NS53. When this region was absent, instead of a cytoplasmic distri-

bution, NS53 accumulated preferentially in association with the nucleus. The conclusion that the intracellular localization domain is located between amino acids 84 to 176 of NS53 is supported by studies on the truncated gene 5 product of the rotavirus variant brvA, which indicated that although it contained only the first 258 amino acids of wild-type NS53, the intracellular distribution pattern of the truncated protein was still like that of the wild-type protein (Fig. 9) (20).

Our IF experiments suggested that the intracellular localization of NS53 was not affected by the presence or absence of any other viral protein, which seemingly excludes the possibility that the association of NS53 with the cytoskeleton is dependent on its interaction with NS34, another rotavirus nonstructural protein that accumulates on the cytoskeleton and possesses RNA-binding activity (30). Given that NS53 appears to recognize the 5' ends of rotavirus mRNA while NS34 recognizes the 3' ends, it is possible that both proteins can interact with the same RNA molecule and thus form a complex on the cytoskeletal network that might serve as an early intermediate in the formation of rotavirus replicase particles (16). On the basis of studies involving the function of VP2, a protein that appears to be an essential component of replication intermediates with replicase activity (29), it seems unlikely that any mRNAs bound to the cytoskeleton are replicated at that site, given that VP2 accumulates in viroplasm. Although speculative, it seems possible that by binding to viral mRNAs, NS53 may serve to block their translation and promote their interaction with the cytoskeleton. The recruitment of mRNA to the cytoskeleton may only require transient interactions of NS53 with the cytoskeleton and thus may explain why significant amounts of NS53 are found both in the soluble and cytoskeletal fractions of infected cells. A role of the cytoskeleton may be to move the mRNA to the viroplasm where the mRNA then interacts with other viral proteins including the RNA-binding protein, NS35 (23), and the structural proteins VP1, VP2, and VP3 to form replication intermediates which have replicase activity (16). Proteins that function as molecular chaperons to specifically localize mRNAs within cells, e.g., *staufen*, have been recognized previously (22, 44). The process of mRNA localization appears to require recognition of localization signals that are present in the untranslated regions of the mRNAs (11, 18). A role for the cytoskeleton network in the binding of mRNA and the intracellular localization of at least some mRNAs, e.g., β -actin, *bicoid*, cyclin B, *gurken*, and Vg1 mRNA, has also been indicated before (22, 33, 52).

Nondefective rotavirus variants have been described that because of gene 5 rearrangements are able to encode only truncated species of NS53 (47). One such mutant, brvA, has been shown to produce in infected cells a carboxyl-truncated NS53 of 258 amino acids (20). Given that the functional domains for RNA binding and intracellular localization reside within the first 176 amino acids of NS53, the truncated NS53 of brvA can be expected to retain these two characteristics (Fig. 9). On the other hand, the rearranged gene 5 of the mutant P9Δ5 appears to encode only the first 150 amino acids of wild-type NS53 (47). As a result, while this truncated species retains the RNA-binding domain, it is not clear whether its pattern of intracellular accumulation would be the same as that of the wild-type protein since the truncation begins within the region of NS53 indicated to include the intracellular localization domain. The ability of rotavirus variants that encode truncated NS53 to replicate in cell culture reveals that the carboxyl half of NS53 is not essential for virus replication at least in vitro. Despite this, two observations suggest that the carboxyl half of NS53 does retain a function that promotes either the rate of virus replication or virus spread. (i) The sizes

of the plaques produced by the rotavirus variants are significantly smaller than that of wild-type virus. (ii) The carboxyl portion of the protein, while poorly conserved among the group A rotaviruses, is highly conserved with respect to secondary structure (20a). It remains to be determined whether NS53, although not required for *in vitro* replication of rotaviruses, is required for the perpetuation of these viruses *in vivo*, possibly serving as a factor that mediates host range or virulence (5).

ACKNOWLEDGMENTS

Vector v2.0 was provided by Asit Pattnaik, and plasmid pUC18-g5 was provided by Gerry Both. The assistance of Luke Jiang in the IF assays is appreciated, and we acknowledge the excellent technical assistance of Wenying Zhao. We are grateful to Gail Wertz for helpful information concerning the use of the vTF7-3 expression system.

This study was supported by grant AI-24478 from the National Institutes of Health. J.T.P. is recipient of Research Career Development Award AI-00990 from the National Institutes of Health.

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