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Rotavirus Protein NSP3 (NS34) Is Bound to the 3′ End Consensus Sequence of Viral mRNAs in Infected Cells

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Interaction between viral proteins and RNAs has been studied in rotavirus-infected cells. The use of UV cross-linking followed by immunoprecipitation and labeling with T4 polynucleotide kinase allowed us to detect interactions between RNA and nonstructural viral proteins. The RNAs linked to the nonstructural protein NSP3 have been identified as rotavirus mRNAs, and the sequences of the RNase T_1-protected fragments have been established. These sequences correspond to the 3′ end sequence common to all rotavirus group A genes. We also show that the last 3′ nucleotide is cross-linked to the protein and that monomeric and multimeric forms of NSP3 are bound to rotavirus mRNA. The role of NSP3 in rotavirus replication is discussed in the light of our results and by comparison with other RNA-binding proteins of members of the *Reoviridae* family.

Rotaviruses are members of the family *Reoviridae*, and their genome is composed of 11 molecules of double-stranded RNA (dsRNA) ranging from 3.3 to 0.6 kb that encode six structural and five nonstructural proteins (10). Virus replication occurs in the cytoplasm, but various steps of this process are not precisely known. As the virus enters the cell, the viral transcriptase is activated and synthesizes capped nonpolyadenylated mRNAs. These viral mRNAs are translated or used as templates for the synthesis of the genomic dsRNAs. Replication is nonconservative; mRNAs are copied in the negative strand, and the dsRNAs thus formed are encapsidated in new viral particles. Encapsidation and replication are concomitant, since dsRNAs are never found free in the cytoplasm but are associated with viral structures. As there is neither solid genetic nor molecular evidence to the contrary, it is generally agreed that each viral particle contains only one set of the 11 genes and only one copy of each gene. One of the most puzzling question about the morphogenesis of reoviridae in general and rotavirus in particular is how the right set of dsRNA segments is encapsidated in the viral particles.

In reoviridae, mRNAs bear 5′ and 3′ untranslated regions of variable length and are bordered by two different sequences common to all genes. In the case of group A rotaviruses, the first four and last five nucleotides are conserved among all segments. The 5′ consensus sequence GGCU(A,U)$_n$ (n = 6 to 9) is more heterogeneous than the 3′ end consensus sequence GUGACC. The latter is highly conserved in the 11 genes except for a slight difference (ΔUGACC) in the four genes 2 sequenced to date. The conservation of these terminal sequences suggests that they are important for transcription, replication, and/or encapsidation of the viral RNAs. The fact that four of the five nonstructural proteins encoded by the virus exhibit RNA-binding properties (2, 3, 15, 17) and are found in intermediate particles with replicase activities (11, 20) substantiates the hypothesis that some rotavirus nonstructural proteins can bind specifically to the conserved sequences. However, none of the viral proteins have been shown to bind to specific sequences of rotavirus mRNAs or dsRNAs in the various tests used. Using a different experimental approach, we have investigated whether, in the course of infection, some of the nonstructural proteins can be bound to viral RNAs. We report here that in rotavirus-infected cells, the nonstructural protein NSP3 is bound to the 3′ conserved end of rotavirus mRNAs.

(The new nomenclature of rotavirus nonstructural proteins proposed at the Fourth International Symposium on Double Stranded RNA Viruses is used throughout the text.)

MATERIALS AND METHODS

Cells, viruses, and antibodies. The RF strain of rotavirus was propagated in MA104 cells in minimum Eagle's medium in the presence of trypsin. Infections were made at a multiplicity of 5 to 10 PFU per cell. In vivo labeling was performed from 0 to 6 h postinfection with 4.10 MBq of Tran-S^{35} label (38 TBq/mol; ICN) per ml. The monoclonal antibody (ID3) raised against recombinant NSP3 will be described elsewhere (1a); the anti-VP6 monoclonal antibody used as control was R50 (21).

UV cross-linking, immunoprecipitation, RNase treatment, and RNA labeling. Six hours postinfection, MA104 cells in 20-cm^2 culture plates were washed with 2 ml of cold Tris-buffered saline (25 mM Tris-HCl [pH 8], 0.7 mM Na_2HPO_4, 5.1 mM KCl, 137 mM NaCl) and exposed on ice to a germicidal UV lamp for 13 min at a 22-cm distance. Tris-buffered saline was removed, and the cells were lysed in 1 ml of radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 8.5], 75 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 20 mM EDTA, 2 mg of aprotinin per ml. Cell debris were pelleted by centrifugation (13,000 × g, 3 min), and the supernatants were processed essentially as described by Stewart et al. (26). Briefly, to immunoprecipitate the RNA-protein complexes, 1 μl of mouse monoclonal ascitic fluid was added to 100 μl of cell lysate and incubated overnight at 4°C. Then 20 μl of a 50% suspension of protein A-Sepharose (Pharmacia) was added, and incubation continued for 1 h at room temperature with end-over-end rotation. Protein A-Sepharose beads were centrifuged (13,000 × g, 10 s) and washed three times with RIPA buffer and twice with RNase T_1 buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% Triton with or without 0.5 M NaCl). RNase T_1 (50 U in

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20 μl of RNase T_1 buffer; Boehringer) was added, and RNA was digested by incubation at 37°C for 30 min. RNA not linked to the protein was eliminated by two washes with RIPA buffer. Protein A-Sepharose beads were washed twice with T4 polynucleotide kinase buffer (50 mM Tris-HCl [pH 8], 1.5 mM spermidine, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol), and RNA linked to the protein was labeled with [γ-^{32}P]ATP (1.85 × 10^9 MBq/mmol) and T4 polynucleotide kinase (3.7 MBq and 10 U, respectively, in 20 μl of T4 polynucleotide kinase buffer per assay) for 20 min at 37°C. Unincorporated [γ-^{32}P]ATP was removed by two washes with RIPA buffer, and beads were processed for protein or RNA analysis (see below).

Nucleic acid analysis, purification, and sequencing. Nucleic acid purification and analysis were carried out according to routine procedures (23). Rotavirus messenger and genomic RNAs were obtained by using standards techniques (5). The rotavirus RF strain cDNA library has been described elsewhere (6), and sequences of genes 1, 2, 5, 6, and 9 have been published and are accessible through the EMBL data base. Sequences of the other genes will be published elsewhere (1a). Plasmid p71, bearing gene 9 with a *Kspl* restriction site at the end of the 3′ noncoding sequence and a polymerase T7 promoter fused to the 5′ noncoding sequence end, was obtained by site-directed mutagenesis (16). A bacterial chloramphenicol acetyltransferase (CAt) gene with *Cla*I and *Bgl*II sites at the 5′ and 3′ ends, respectively, was amplified from pSV2Cat (12) by polymerase chain reaction using appropriate primers and then cloned in place of the gene 9 coding sequence in p71 to obtain plasmid p71Cat. The clone thus obtained contains the complete 5′ and 3′ untranslated regions of rotavirus gene 9 (48 and 32 nucleotides, respectively) with parts of the coding sequence (50 and 44 nucleotides of the 5′ and 3′ ends, respectively).

For RNA sequencing, bands identified by autoradiography were cut from the polyacrylamide-urea gel and crushed in 0.5 M ammonium acetate (pH 5.5)–1 mM EDTA. After elution, RNAs were purified by two successive chromatographies on Sephadex G-10 and G-25. Sequencing was done by the enzymatic technique (8, 9) as instructed by the kit supplier (U.S. Biochemical) except that digestions were done at 55°C and alkaline degradation was done at 90°C. After digestion with nuclease P1 (1,000 cpm of RNA and 1 μg of P1 nuclease in 25 mM ammonium acetate buffer [pH 5.3]), 5′ end nucleotides were determined by thin-layer chromatography on polyethyleneimine-cellulose plates; solvents were 1 M acetic acid and then 0.3 M LiCl (22).

Nucleic acid blotting and hybridization. DNA digests were separated by electrophoresis on 1.5% agarose gels and transferred in denaturing buffer (0.4 N NaOH, 1.5 M NaCl) to a nylon membrane (Nytran; Schleicher & Schuell), neutralized with 1 M Tris-HCl (pH 8)–1.5 M NaCl, and then cross-linked by UV irradiation.

RNA was denatured by incubation at 70°C for 2 min in gel loading buffer (1× TBE [0.089 M Tris-HCl, 0.089 M boric acid, 2 mM EDTA], 0.01% bromophenol blue) containing 80% formamide and then separated by electrophoresis on 1.5% agarose gels. Transfer to a Hybond N^+ (Amersham) membrane was done in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RF cDNA genes (cloned in plasmid pBS [Stratagene]) spotted on a nitrocellulose filter were denatured by incubation in denaturing buffer, neutralized with 1 M Tris-HCl (pH 8)–1.5 M NaCl, and then baked for 2 h at 80°C. Membrane-bound nucleic acids were prehybridized in 4× SSC–5× Denhardt solution (Ficoll, polyvinylpyrrolidone, bovine serum albumin; 1% [wt/vol])–50 μg of *Escherichia coli* tRNA per ml for 2 h at 60°C. Before hybridization, the temperature of the water bath was raised to 90°C, the probe was denatured for 3 min at 80°C, and hybridization (150,000 cpm of probe per ml) was allowed to proceed by cooling the water bath to 30°C. Membranes were then washed twice in 4× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature before exposure.

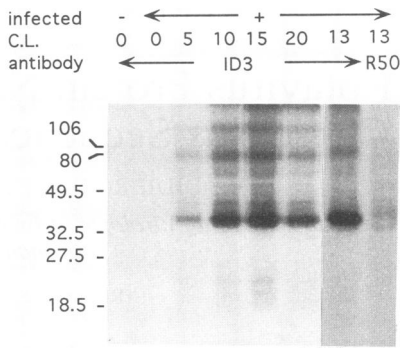

FIG. 1. NSP3-RNA UV cross-linking. Lysates from rotavirus-infected (+) or mock-infected (−) MA104 cells were subjected to UV cross-linking (C.L.) for the indicated times (in minutes), immunoprecipitated with an anti-NSP3 (ID3) or anti-VP6 (R50) monoclonal antibody, and processed as described in Materials and Methods. Sizes of the molecular weight markers are indicated in kilodaltons.

Protein analysis. Immunoprecipitated proteins were resolved on a 12% polyacrylamide gel after boiling in loading buffer (10 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 150 mM 2-mercaptoethanol). For NSP3 multimer analysis, 2-mercaptoethanol was omitted. After electrophoresis, gels were fixed in 20% methanol–10% acetic acid, dried, and autoradiographed. When in vivo ^{35}S-labeled proteins were analyzed, gels were treated with Amplify (Amersham) before drying and autoradiography.

RESULTS

Cross-linking of NSP3 to RNA. To investigate the RNA-binding properties of NSP3, we used a method first described by Meric (18) whereby the RNAs bound to the protein are cross-linked by UV light and the RNA-protein complexes are immunoprecipitated with a specific antibody. Successive RNase T_1 digestion and 5′ end RNA labeling are carried out on the RNA-protein complexes bound to the protein A-Sepharose beads.

To determine the experimental conditions optimal for cross-linking, infected cells were subjected to increasing times of UV irradiation. Experiments were performed at 6 h postinfection, the time at which the replication/transcription ratio is maximal (25). Under such conditions (Fig. 1), one major protein band of about 40 kDa and two faint bands of 80 and 135 kDa were immunoprecipitated with the anti-NSP3 monoclonal antibody. The amount of ^{32}P radioactivity associated with those bands increased up to 15 min of cross-linking. We later used a suboptimal 13-min dose of UV irradiation to minimize nonspecific cross-linking and damage to proteins or RNAs.

The presence of the 40-kDa band was strictly dependent on infection by rotavirus and on use of the anti-NSP3 monoclonal antibody (Fig. 1). This assay identified the 40-kDa protein as the rotavirus NSP3 protein (the other two bands were identified as multimers of NSP3; see below).

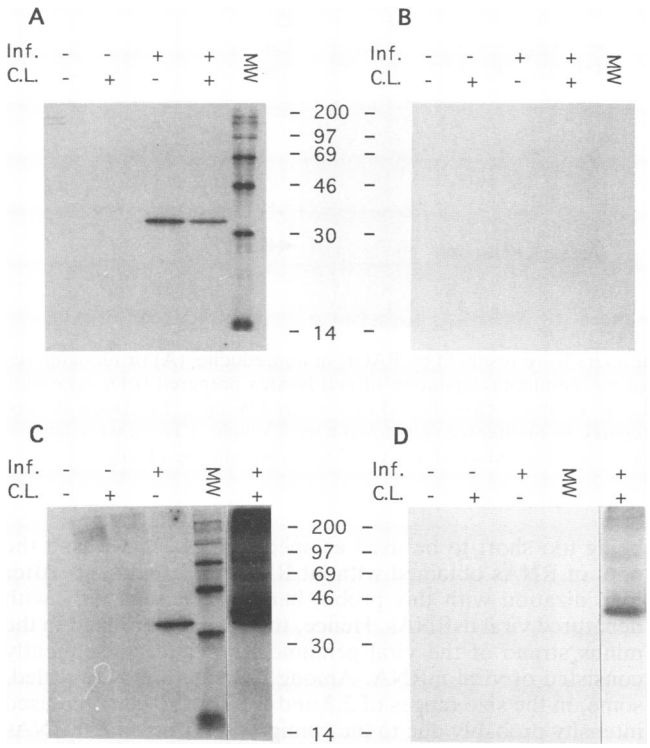

FIG. 2. Specific labeling of NSP3-RNAs complexes. Mock-infected (Inf. −) or rotavirus-infected (Inf. +) MA104 cells were labeled with [^{35}S]methionine for 6 h postinfection and either not cross-linked (C.L. −) or cross-linked (C.L. +) for 13 min. Cells lysates were immunoprecipitated with the anti-NSP3 monoclonal antibody and processed as described in Materials and Methods (C and D); in control experiments (A and B), T4 polynucleotide kinase was omitted from the labeling mix. Two X-ray films separated by aluminum foil were put on the dried gels. Panels A and C are from the film in contact with the gel and show the ^{35}S-, ^{32}P-, and ^{14}C-labeled bands; panels B and D are from the upper film and show only ^{32}P-labeled bands. The sizes of the ^{14}C-labeled molecular weight markers (MW) are indicated in kilodaltons.

Moreover, labeling of NSP3 with [γ-^{32}P]ATP was obtained only after UV cross-linking and addition of T4 polynucleotide kinase in the labeling reaction (Fig. 2). These results ruled out the possibility that a kinase coprecipitated (or was cross-linked) with NSP3 or that NSP3 was itself a kinase or an ATP-binding protein.

When immune complexes were treated with RNase A (0.1 μg/20 μl) instead of RNase T_1, the radioactivity associated with NSP3 was greatly reduced (Fig. 3). On the other hand, treatment with RNase T_1 in a high-salt buffer, which stabilizes double-stranded regions of RNAs, did not affect the intensity of the band (Fig. 3). These results suggested that a single-stranded RNA was cross-linked to NSP3. Furthermore, when the in vivo ^{35}S-labeled NSP3 was run in parallel with the cross-linked in vitro ^{32}P-labeled protein (Fig. 2; see also Fig. 4), the latter migrated slightly more slowly; these differences in migrations very likely were induced by a short oligoribonucleotide linked to the protein.

Multimeric forms of NSP3. As NSP3 forms multimers when expressed either in recombinant baculovirus-infected Sf9 cells or in rotavirus-infected MA104 cells (17) and binding of proteins to nucleic acids is achieved in some cases by multimerization (19), we investigated whether NSP3 was bound to RNAs as a monomer or multimer.

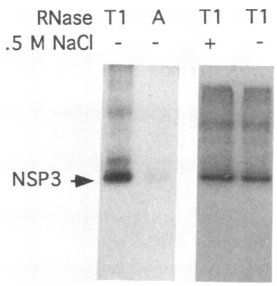

FIG. 3. Various RNase treatments of NSP3-RNAs complexes. Lysates from infected, UV-cross-linked MA104 cells were processed as described in Materials and Methods except that RNase T_1 was substituted by RNase A and/or the RNase buffer was adjusted to 0.5 M NaCl.

When NSP3 cross-linked to RNA and ^{32}P labeled was analyzed by polyacrylamide gel electrophoresis (PAGE) without reduction by 2-mercaptoethanol (Fig. 4), two additional bands in the size range of 80 and 135 kDa were observed. The presence of some immunoprecipitated material that did not penetrate the separating gel suggested that higher complexes of NSP3 were also present. In addition, with the cross-linked material, the 80- and 135-kDa bands were slightly visible in reducing conditions. These faint bands are very likely the result of protein-protein cross-linking. In the same conditions, immunoprecipitation of in vivo ^{35}S-labeled NSP3 led to a similar pattern of bands (Fig. 4). The only noticeable difference was that after UV cross-linking, monomeric NSP3 migrated as a doublet. As mentioned above, the higher (40-kDa) band of the doublet corresponded to the RNA-cross-linked NSP3, as it could be labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Fig. 4), whereas the lower (35-kDa) band that comigrated with the non-cross-linked NSP3 corresponded to the free protein. Together, these results demonstrated that the 80- and 135-kDa bands are homomultimers of NSP3. According to their apparent molecular weights, they correspond to dimers and trimers or tetramers of NSP3. The sensitivity of multimers to 2-mercaptoethanol and, to a lesser degree, temperature suggested multimerization of NSP3 through disulfide bond formation and hydrophobic interactions.

Characterization of the RNAs cross-linked to NSP3. To recover the RNAs linked to NSP3, immune complexes were treated with proteinase K, and the RNAs thus freed from their protein moiety were analyzed on denaturing polyacrylamide gels (Fig. 5). In the absence of experimental RNase treatment, the NSP3-bound RNAs migrated as a set of discrete bands in the size range of 10 to 50 nucleotides; they may have originated from nonspecific cellular RNase digestion in the course of cell lysis and from breaks induced by UV light. When treatments with increasing concentrations of RNase T_1 were performed, the pattern of RNAs bound to NSP3 was reduced to four major and two minor bands with apparent sizes of 9 to 15 nucleotides. When the RNase T_1 treatment was done in the presence of 0.5 M NaCl (Fig. 5), two additional bands, 23 and 18 nucleotides long, were observed. That pattern will serve as a reference, and RNA fragments will be referred to as bands 1 to 8 (from bottom to top). In contrast to the simple pattern of bands obtained with NSP3-bound RNAs, RNase T_1-treated rotavirus mRNAs showed a ladder of bands from 1 to 45 nucleotides long. This finding implied that NSP3 was linked to a rather homogeneous population of single-stranded RNAs and protected a short stretch of RNA.

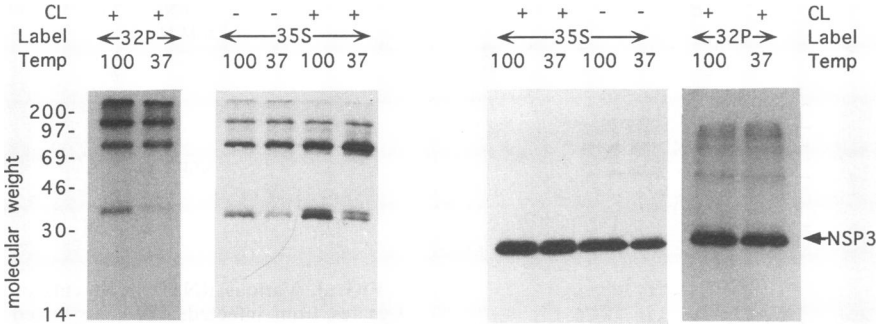

FIG. 4. Multimeric forms of native and RNA-bound NSP3. NSP3 multimers were resolved by PAGE in nonreducing (A) or reducing (B) conditions. For lanes marked Label 35S, ^{35}S-labeled NSP3 was obtained by immunoprecipitation of cell lysates prepared from rotavirus-infected cells labeled in vivo with [^{35}S]methionine and then cross-linked (CL +) or not cross-linked (CL −) by UV light. For lanes marked Label 32P, multimers, obtained by immunoprecipitation of cell lysates prepared from infected cells treated by UV light (CL +), were labeled in vitro with [γ-^{32}P]ATP and T4 polynucleotide kinase. Immune complexes were incubated at 100 or 37°C before loading (Temp).

Identification of NSP3-bound RNAs. To identify the NSP3-bound RNAs, we first used these RNAs as probes against viral mRNAs, denatured viral dsRNAs, and unrelated RNAs (Fig. 6A). The RNAs obtained after RNase T_1 treatment being too short to be used as specific probes, we used the pool of RNAs obtained without RNase T_1 treatment. After hybridization with this probe, bands were seen only with denatured viral dsRNAs. Hence, the probe hybridized to the minus strand of the viral genomic RNA and consequently consisted of viral mRNA. Among the nine bands revealed, some, in the size ranges of 2.3 and 1.3 kb, showed increased intensity probably due to the comigration of several mRNAs and suggested the presence of a complete set of viral mRNA in the probe. The presence of the 11 rotavirus mRNAs in the probe was further confirmed by hybridization of dot spots of the 11 cloned cDNAs (Fig. 6B). It should be emphasized that the intensity of the bands (Fig. 6A) and of the spots (Fig. 6B) did not necessarily reflect the quantity of each viral mRNA bound to NSP3 in the cell; the efficiency of UV cross-linking and of labeling and the length of the cross-linked RNA can differ from one mRNA to another and thus impair quantitation.

These results showed that the 11 minus-strand rotavirus RNAs could hybridize to the probe and hence that the

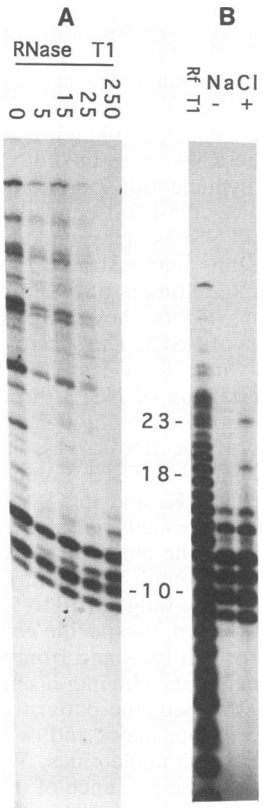

FIG. 5. Analysis of RNAs cross-linked to NSP3. Shown are results of denaturing PAGE (20% polyacrylamide gel) of RNA purified from RNA-NSP3 complexes treated with increasing concentrations of RNase T_1 in standard buffer (A) or with 50 U of RNase T_1 in standard or 0.5 NaCl buffer (B). RF T1 corresponds to rotavirus mRNA digested to completion with RNase T_1 before 5′ end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP. RNA length (in nucleotides) was established by comparison with a synthetic RNA of defined sequence (U.S. Biochemical).

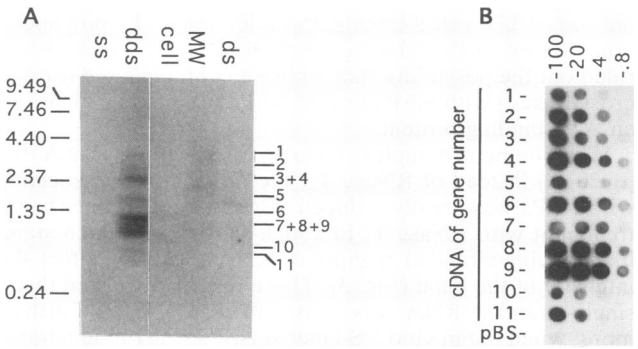

FIG. 6. NSP3-bound RNAs as probes. (A) Native (ds) or denatured (dds) rotavirus genomic RNA, rotavirus mRNA (ss), MA104 total RNA (cell), and RNA molecular weight standards (MW) were resolved on a 1.5% agarose gel, transferred to a membrane, and hybridized with NSP3-cross-linked labeled RNAs. Sizes (in kilobases) of the RNA molecular weight standards and gene numbers are indicated on the left and right, respectively. Ethidium bromide staining of the gel revealed similar amounts of viral mRNA and denatured dsRNA. (B) Dot spots of 100, 20, 4, and 0.8 ng of cloned cDNA of the rotavirus RF genes (1 to 11) and wild-type vector (pBS) were hybridized with NSP3-cross-linked labeled RNAs.

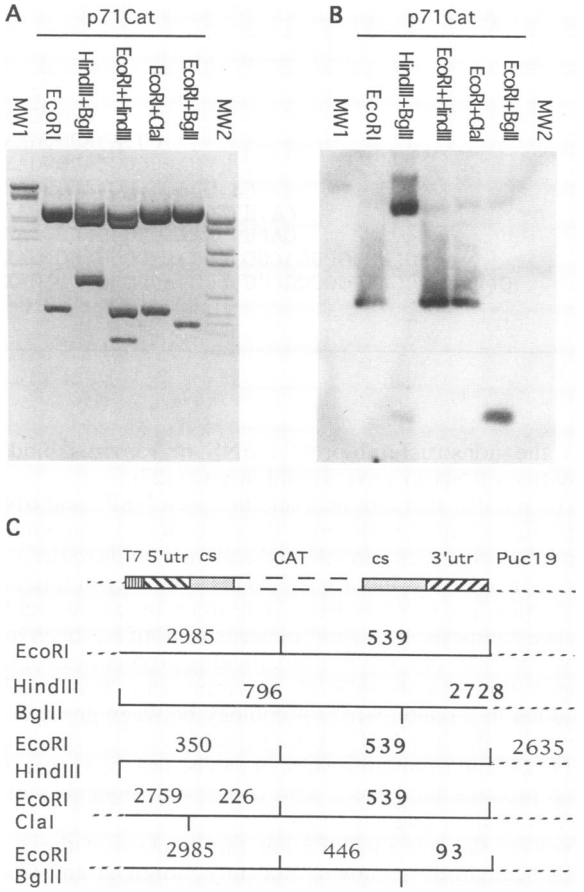

FIG. 7. NSP3-bound RNA as probes on a rotavirus cDNA target. Plasmid p71Cat was digested with the indicated restriction enzymes and hybridized to NSP3-bound RNAs. (A) Ethidium bromide-stained gel; (B) autoradiogram; (C) restriction map of p71Cat. Sizes (in base pairs) of the hybridized bands are indicated in bold; RF gene 9 untranslated regions (utr) and coding sequences (cs) and pUC19 and T7 promoter sequences are indicated on each side of the CAT gene. Molecular weight standards (Boehringer): MW1, lambda phage DNA *Eco*RI-*Hin*dIII digest (21,226, 5,148, 4,973, 4,268, 3,530, 2,027, 1,904, 1,584, 1,375, 947, 831, and 564 bp); MW2, pBR328 *Bgl*I and pBR328 *Hin*fI digests (2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, and 298 bp).

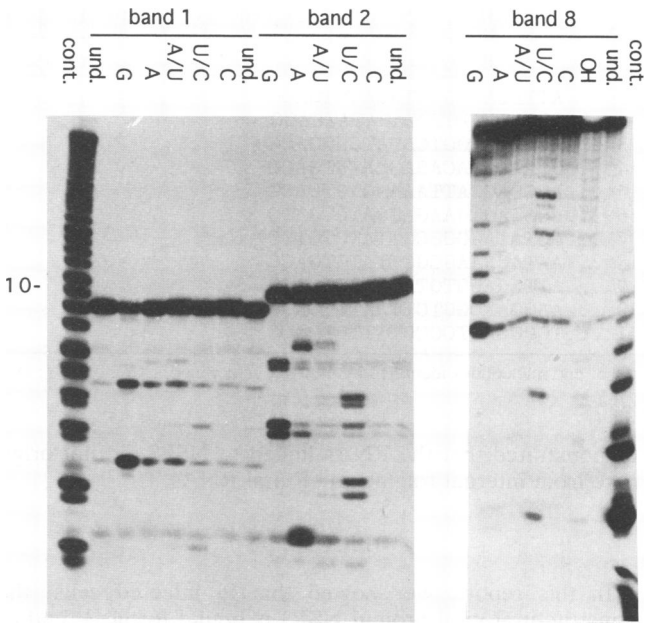

FIG. 8. Sequences of NSP3-cross-linked RNA bands 1, 2, and 8. Partial digests of 5′-end-labeled RNA were obtained with RNases T_1 (lanes G), U2 (lanes A), and phy M (lanes A/U), *B. cereus* RNase (lanes U/C), and RNase CL3 (lanes C). OH, partial alkaline degradation; und., undigested RNA; cont., partial alkaline degradation of a 30-nucleotide-long synthetic RNA (U.S. Biochemical).

NSP3-bound RNAs contained sequences included in each of the 11 rotavirus mRNAs. To determine whether these sequences were present at the 3′ or 5′ ends of the rotavirus genes, the same probe was used to hybridize restriction digests of a DNA clone (p71Cat) bearing the conserved 3′ and 5′ sequences of a rotavirus gene (Fig. 7). Only the fragments containing the 3′ end of the rotavirus gene were hybridized; thus, the RNAs cross-linked to NSP3 originate from the 3′ ends of rotavirus mRNAs.

Sequencing of the NSP3-bound RNAs and localization of the cross-linked nucleotide. We further characterized the RNAs protected by NSP3 by purifying the eight bands obtained after RNase T_1 digestion in 0.5 M NaCl buffer and sequencing them (Fig. 8 and Table 1). Although two-dimensional gel electrophoresis (data not shown) and thin-layer chromatography analysis (Table 1) showed that some bands were composed of more than one RNA fragment, all of the RNAs sequence ladders showed the presence of the same sequence, ...UGUGAxx, at their 3′ ends (Fig. 8 and Table 1). This sequence matched the 3′ end conserved sequence of rotavirus RNAs (Table 1). The shortest band protected by NSP3 corresponded to the most frequent 3′ end sequence, and the other bands, despite some ambiguities due mainly to lack of digestion by the CL3 RNase, corresponded to the same sequence extending in the 5′ direction. In addition, the sequences of bands 7 and 8 could be attributed to the last 17 and 22 nucleotides of genes 8 and 7, respectively. It is probable that these two mRNAs present a particular secondary structure at their 3′ ends, as bands 7 and 8 appeared after RNase T_1 digestion in high-salt buffer (Fig. 5).

A common characteristic of the eight RNAs was that upon partial alkaline degradation, the penultimate fragment migrated two nucleotides shorter than the full-length fragment and one nucleotide longer than the largest fragment obtained with RNase U2 (Fig. 9). Similar gaps have been previously observed with cross-linked material and are attributable to the amino acid covalently linked to the nucleic acid under UV irradiation (4). The position of this gap clearly established that NSP3 was cross-linked to the last nucleotide of the RNA and that one or two amino acids were not removed by the proteinase K treatment. If NSP3 was internally cross-linked to the RNAs, nucleotides present downstream the cross-link site would have changed the position of the gap present on the partial alkaline degradation ladder. Furthermore, cross-linking of the last nucleotide to the protein could hinder hydrolysis of the penultimate nucleotide by CL3 or *Bacillus cereus* RNase (Fig. 8) and is consistent with the inability to label the 3′ end of the RNAs with [^{32}P]pCp and RNA ligase (data not shown).

The terminal position of the sequence shared by the NSP3-linked RNAs and of the cross-link site on RNAs

TABLE 1. Nucleotide sequences of 3′ ends of strain RF genes and ribonucleotide sequences of NS34-bound RNAs

Gene no. and sequence	Bands sequences and no.[a]
1 ...ATTAGAGCGCTTAGATGTGACC	
2 ...ACCCCACTGTGGAGATATGACC	AUGUGAXX 1
3 ...ACACACTGGTCACATCGTGACC	(U,A)AUGUGAXX 2
4 ...GCAACTGACAGAGGATGTGACC	U(UC)AUGUGAXX 3
5 ...AGGCAGCATTAAAAGTGTGACC	(A,C)A(A,UC)AUGUGAXX 4
6 ...GCGTAGTGAAGAGGATGTGACC	(A,U)X(UC)AAA(UC)GUGAXX 5
7 ...GCTATAGGGGCGTTATGTGACC	CA(UC)AAAA(UC)G(UC)GAXX 6
8 ...AATAGAAAGCGCTTATGTGACC	AAAG(UC)G(UC)(UC)(UC)A(UC)G(UC)GAXX 7
9 ...ATTAGAGGTGTACGATGTGACC	C(UC)A(UC)AGGGGCG(UC)(UC)A(UC)G(UC)GAXX 8
10 ...AAGGAACGGTCTTAATGTGACC	
11 ...TCCCCACTCGCGTTTTGTGACC	

[a] 5′ end nucleotides identified by TLC are underlined. (XY), X or Y; (X,Y), X and Y.

demonstrated that the RNAs linked to NSP3 did not originate from internal fragments of viral RNAs.

DISCUSSION

In this report, we showed that in infected cells, the nonstructural viral protein NSP3 is bound to the 3′ end of rotavirus mRNA and protects the whole 3′ end consensus sequence from RNase T_1 digestion. Moreover, hybridization experiments showed that the mRNAs linked to NSP3 correspond to the 11 rotavirus genes.

Nonstructural proteins have been found associated with single-stranded RNAs in orthoreoviruses (1, 27) and rotaviruses (2, 3, 15, 17), but few specific RNA-binding proteins have been identified in reovirus-infected cells. A host factor binding to the terminal sequences of viral RNAs of a phytoreoviridae has been detected (7), and it has been shown that the nonstructural protein σNS of reovirus binds to reovirus mRNA in vivo (1) and in vitro (27).

The parallel that can be made between NSP3 and σNS is of particular interest because the latter forms homomultimeric 13S-19S particles in infected cells and binds to the 3′ end of reovirus mRNA in vitro (27). This similarity in functional properties has recently been reinforced by amino acid sequence comparison. A short consensus motif has been found between rotavirus NSP3, RNA-binding protein NS2 of bluetongue virus, and σNS of reovirus (28). This motif is included in a region where homology between group A and group C rotavirus NSP3 proteins is high (17). From earlier work (27) and from our results, it can be proposed that these three proteins bind the 3′ end conserved sequences of the mRNA of their cognate virus.

Neither the role of NSP3 nor the function of the 3′ end RNA sequences is known. Recently Gorziglia and Collins (13) have shown that deletion of the 3′ end consensus sequence considerably reduces the replication and expression of rotavirus-like RNA transfected in rotavirus-infected cells. With the results described here, it is tempting to assume that such deletions impair the binding of NSP3 to rotavirus RNAs. The binding of NSP3 could lead to rotavirus RNA expression in different ways. For instance, NSP3 can play the role of the poly(A) sequences in protecting viral mRNA from degradation and in regulating translation (14). NSP3 can also be involved in the transport of viral mRNAs from the transcribing particles to the site of replication. Cellular structures and 3′ end sequences of mRNA are involved in mRNA localization (24), and immunofluorescence studies have detected NSP3 associated in a complex network throughout the cell cytoplasm (17). These hypotheses are not exclusive of a role of NSP3 in the early steps of rotavirus mRNA replication. For example, the selective binding of NSP3 to the 3′ conserved sequence would allow NSP3 to select rotavirus mRNAs among cellular RNAs and to collect them. The involvement of NSP3 in the selection process leading to the encapsidation of the right set and the right molar ratio of genes remains obscure because NSP3 is bound to a sequence common to all rotavirus genes. The minimal RNA sequence essential for binding of NSP3 to viral mRNA should be studied in vitro in order to establish whether NSP3 is able to fulfill this role alone or in association with some other viral protein(s).

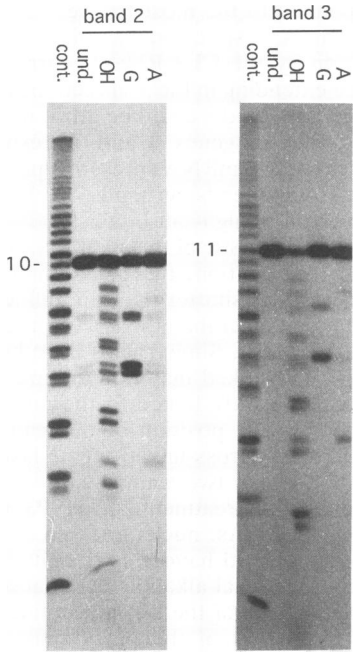

FIG. 9. Location of the RNA-protein cross-link site. Partial digests of 5′-end-labeled RNA were obtained with RNases T_1 (lanes G) and U2 (lanes A). OH, partial alkaline degradation; und., undigested RNA; cont., partial alkaline degradation of a 30-nucleotide-long synthetic RNA (U.S. Biochemical).

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