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Rotavirus VP1 Alone Specifically Binds to the 3′ End of Viral mRNA, but the Interaction Is Not Sufficient To Initiate Minus-Strand Synthesis

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Recent studies have shown that disrupted (open) rotavirus cores have an associated replicase activity which supports the synthesis of dsRNA from viral mRNA in a cell-free system (D. Chen, C. Q.-Y. Zeng, M. J. Wentz, M. Gorziglia, M. K. Estes, and R. F. Ramig, J. Virol. 68:7030–7039, 1994). To determine which of the core proteins, VP1, VP2, or VP3, recognizes the template mRNA during RNA replication, SA11 open cores were incubated with ^{32}P-labeled RNA probes of viral and nonviral origin and the reaction mixtures were analyzed for the formation of RNA-protein complexes by gel mobility shift assay. In mixtures containing a probe representing the 3′ end of SA11 gene 8 mRNA, two closely migrating RNA-protein complexes, designated *s* and *f*, were detected. The interaction between the RNA and protein of the *s* and *f* complexes was shown to be specific by competitive binding assay with tRNA and brome mosaic virus RNA. By electrophoretic analysis of RNA-protein complexes recovered from gels, VP1 was shown to be the only viral protein component of the complexes, thereby indicating that VP1 specifically recognizes the 3′ end of gene 8 mRNA. Analysis of VP1 purified from open cores by glycerol gradient centrifugation verified that VP1 recognizes the 3′ end of viral mRNA but also showed that in the absence of other viral proteins, VP1 lacks replicase activity. When reconstituted with VP2-rich portions of the gradient, VP1 stimulated levels of replicase activity severalfold. These data indicate that VP1 can bind to viral mRNA in the absence of any other viral proteins and suggest that VP2 must interact with the RNA-protein complex before VP1 gains replicase activity.

The rotaviruses, members of the family *Reoviridae*, are the major cause of acute infantile diarrhea worldwide, causing nearly one million deaths annually (2). The rotavirion is an icosahedral triple-layered particle that contains a genome of 11 segments of double-stranded RNA (dsRNA) (25, 29). Together with the genome, the proteins VP1, VP2, and VP3 of the innermost layer form the core of the virion (1). Indirect evidence suggests that one of the minor core components, VP1, is the viral RNA-dependent RNA polymerase: (i) VP1 contains sequence motifs shared by RNA-dependent RNA polymerases of other viruses (6, 7, 18), (ii) VP1 binds nucleotides and cross-linking of the nucleotide analog azido-ATP to VP1 inhibits viral RNA polymerase activity (26), and (iii) baculovirus-expressed core-like particles (CLPs) consisting of VP1 and VP2 can catalyze the synthesis of dsRNA from template mRNA (replicase activity), but CLPs consisting of only VP2 lack such polymerase activity (31). VP3, the other minor protein component of the core, covalently binds GTP and has been proposed to function as the viral guanylyltransferase (7, 14, 22). VP2, the major protein component of the core, has nonspecific RNA-binding activity for both single-stranded RNA and dsRNA and is able to self-assemble into CLPs (3, 11, 12). Although VP2 CLPs have been shown by assay in cell-free systems to lack replicase activity (31), studies with rotavirus temperature-sensitive mutants have indicated that VP2 plays an essential role in the formation of replication intermediates with replicase activity (15).

Rotavirus mRNA is capped but nonpolyadenylated (9, 16) and functions both to direct protein synthesis and as the template for the synthesis of dsRNA in infected cells (4, 19). Chen et al. (4) have established a template-dependent cell-free system that supports the synthesis of rotavirus dsRNA from input mRNA. These investigators included as a source of replicase activity in the system either virion-derived cores or baculovirus-expressed VP1-VP2-VP3 CLPs that had been disrupted ("opened") by incubation with hypotonic buffers. Subsequently, the system was used to define the locations of *cis*-acting signals that promote minus-strand synthesis in rotavirus gene 8 and 9 mRNAs (21, 27, 28). A signal essential for replication was mapped to the extreme 3′ ends of gene 8 and 9 mRNAs and, because of the highly conserved nature of its sequence, is probably a common feature of all 11 rotavirus mRNAs (21). In the study described herein, a mobility shift assay and the cell-free replication system were used to identify and compare the protein components of open cores that recognize the 3′ end of viral mRNA with those that are required for replicase activity. The data indicate that while VP1 is able to specifically bind to viral mRNA in the absence of other viral proteins, the interaction between VP1 and the mRNA does not lead to the formation of a complex with replicase activity. Instead, other viral proteins are also required to initiate the synthesis of dsRNA.

MATERIALS AND METHODS

Preparation of open cores. MA104 cells were grown in Eagle's minimal essential medium containing 10% fetal bovine serum. Simian rotavirus SA11 was propagated in and purified from MA104 cells as described before (21). The conditions used to prepare open cores from SA11 virions have been published previously (21). To produce ^{35}S-labeled cores, SA11-infected cells were maintained in 80% Met-Cys-free Eagle's minimal essential medium containing 25 μCi of ^{35}S-labeled amino acids (>100 Ci/mmol; NEN) per ml.

Synthesis of RNA probes. The template used to produce the 43-base 3′-v RNA probe was generated by PCR under the following conditions: 1 min at 94°C, 2

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min at 45°C, and 2 min at 72°C (40 cycles) (5). The amplification reaction mixtures contained 2.5 U of *Taq* DNA polymerase, 1 μM concentrations of the oligonucleotide primers T7g8R1016P and PCRg8R3GG, and 0.25 μg of plasmid SP65g8R. The sequence of T7g8R1016P was taatacgactcactataGGATGATGAT GGCTTAGC, and that of PCRg8R3GG was GGTCACATAAGCGCTTTCT AT; the virus-specific sequences are in uppercase letters, while the T7 promoter sequence is in lowercase letters. The PCR product was treated with T4 DNA polymerase to remove overhang A residues and purified by electrophoresis on a 1.5% low-melting-point agarose gel. The T7 transcription template used for the synthesis of the 48-base nonviral (nv) RNA probe was produced by cleaving SP72 (Promega) with *Sma*I. The template used for the synthesis of the 48-base 5′-v RNA probe was generated by cleaving the gene 8 cDNA of SP65g8R at position 45 with *Sty*I (21).

RNA probes were synthesized by runoff transcription with the Ambion T7-MEGAshort transcription system. Reaction mixtures contained 1 to 5 μg of the appropriate template DNA, 7.5 mM (each) GTP, CTP, ATP, and UTP, and 35 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham) and were incubated overnight at 37°C (17). Following transcription, the DNA templates were removed by treatment with RNase-free DNase and the RNA was deproteinized by phenol-chloroform extraction and recovered by ethanol precipitation. Transcripts were resolved by electrophoresis on an 8% polyacrylamide gel containing 6 M urea, and the ^{32}P-labeled RNA probe band was identified by autoradiography. The portion of the gel containing the probe was excised and incubated overnight in water. The eluted probe was recovered by ethanol precipitation and resuspended in water. The sequences of the RNA probes are as follows: 3′-v, GGATGATGA TGGCTTAGCAAGAATAGAAAGCGCTTATGTGACC; 5′-v, GGCTTTTA AAGCGTCTCAGTCGCCGTTTGAGCCTTGCGTGTAGCCATG; and nv, GGGAGACCGGCAGATCTGATATCATCGATGAATTCGAGCTCGGTA CCC.

Gradient separation of core components. One hundred microliters of ^{35}S-labeled open cores was incubated with 5 μg of leupeptin, to inhibit protease activity, and 10 μg of unlabeled 3′-v probe, to stimulate the release of VP1 from the cores. After incubation for 3 h at room temperature, 2 μl of mouse anti-VP6 monoclonal antibody 1026 was added and the sample was incubated at 4°C for 1 h. The 1026 antibody was kindly provided by Pierre Pothier and was included in the sample to aggregate the residual VP6 in the open core preparations. The sample was diluted to 500 μl with low-salt buffer (LSB) (2 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 0.6 mM dithiothreitol) and layered onto a 12-ml gradient of 10 to 30% (vol/vol) glycerol in LSB. The gradient was centrifuged for 18 h at 32,000 rpm in a Beckman SW40 rotor at 4°C, and 0.5-ml fractions were collected. The pellet of the gradient was resuspended in 0.5 ml of LSB. The distribution of ^{35}S-labeled protein in the gradient was analyzed by electrophoresis of 75-μl aliquots of each fraction and the pellet on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels (13). The gels were dried, and ^{35}S-labeled protein was detected by autoradiography.

Mobility shift assay. Typically, reaction mixtures contained 6 to 12 μg of open cores in LSB and 0.2 to 5 μg of RNA probe (specific activity, approximately 50,000 cpm/μg) in a final volume of 10 to 25 μl. To assess the specificity of RNA-protein interactions, 0.5 μg of brome mosaic virus RNA (Promega) or 1.5 to 8 μg of yeast tRNA was included in some reaction mixtures as competitor RNAs. Reaction mixtures were incubated for 30 min at room temperature, and then 0.5 volume of sample buffer (20% glycerol in LSB, 0.1% bromphenol blue) was added. RNA-protein complexes in the samples were resolved by electrophoresis on nondenaturing 8% polyacrylamide gels containing 50 mM Tris-glycine, pH 8.8 (10). Electrophoresis was performed at 250 V until 15 min after the bromphenol blue dye front had migrated off the bottom of the gel. The gels were then dried and subjected to autoradiography. Band intensities were measured with a Scanmaster 3+ densitometer and Bio Image Whole Band Analyzer package, version 3.2.

The mobility shift assay was modified to detect RNA-binding proteins in fractions collected from glycerol gradients. Aliquots (50 μl) of the fractions were incubated with 1 μg of ^{32}P-labeled 3′-v probe, and the samples were allowed to incubate for 60 min at room temperature. Afterwards, the samples were subjected to electrophoresis on nondenaturing polyacrylamide gels as described above.

Replicase assays. Following sedimentation of open cores on 10 to 30% glycerol gradients, fractions of the gradient were assayed for replicase activity with the template-dependent cell-free replication system developed by Chen et al. (4). The components of reaction mixtures were the same as described elsewhere (21) except that 10-μl portions of gradient fractions were added in place of open cores. As the template for replication, 1 μg of gene 8 mRNA was included in reaction mixtures. Newly synthesized RNA was radiolabeled by including 15 μCi of [^{32}P]UTP (800 Ci/mmol) in each reaction. Reaction mixtures were incubated for 3 h at 37°C, and the ^{32}P-labeled gene 8 dsRNA product was detected by electrophoresis on SDS-polyacrylamide gels (21). The gene 8 mRNA added to reaction mixtures was generated by runoff transcription of *Sac*II-cleaved SP65g8R (21).

RESULTS

RNA-protein complexes formed with open cores. Of the three protein components of open cores, i.e., VP1, VP2, and

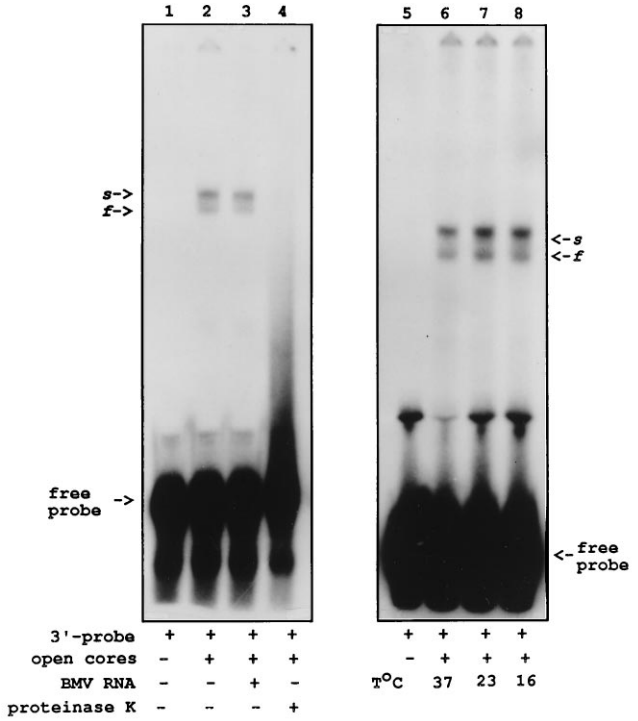

FIG. 1. RNA-protein complexes formed with a gene 8 3′-terminal RNA probe. ^{32}P-labeled 3′-v probe was used at 0.25 μg (lanes 1 to 4) or 0.16 μg (lanes 5 to 8), brome mosaic virus (BMV) RNA (Promega) was used at 0.5 μg, and proteinase K was used at 25 μg. RNA-protein complexes in the samples were detected by electrophoresis on nondenaturing 8% polyacrylamide gels and by autoradiography.

VP3, one or more must specifically recognize the essential *cis*-acting replication signal located at the 3′ end of rotavirus mRNA. To identify the protein(s), a ^{32}P-labeled RNA probe that contained the last 43 bases of gene 8 mRNA (3′-v probe) was produced. The labeled probe was then incubated with open cores, and the mixture was assayed for the formation of RNA-protein complexes by a gel mobility shift assay (Fig. 1). In contrast to results for control reaction mixtures lacking core proteins (Fig. 1, lane 1), two closely migrating RNA-protein complexes designated *s* (slow) and *f* (fast) were detected in reaction mixtures that contained core proteins and the 3′-v probe (lane 2). In this and all other experiments in which the 3′-v probe was used, the amount of the *s* complex detected by mobility shift assay was generally greater than or equal to the amount of the *f* complex detected. Upon addition of proteinase K (Fig. 1, lane 4) and RNase (not shown) to reaction mixtures, the *s* and *f* complexes were not recovered, confirming that these complexes contained RNA and protein components. Both the *s* and *f* complexes were formed over a range of temperatures, including 37°C, the temperature at which open cores are assayed for replicase activity in the cell-free system (Fig. 1, lanes 6 to 8) (4). The possibility of a precursor-product relationship between the *s* and *f* complexes was investigated by incubating open cores with ^{32}P-labeled 3′-v RNA for periods ranging between 5 min and 3 h and then analyzing the reaction mixtures by gel mobility shift assay. The results showed that both the *s* and *f* complexes formed within 5 min of incubation and that the ratio of the *s* complex to the *f* complex was not significantly changed as a factor of the length of incubation (data not shown).

To assess the stability of the *s* and *f* RNA-protein complexes,

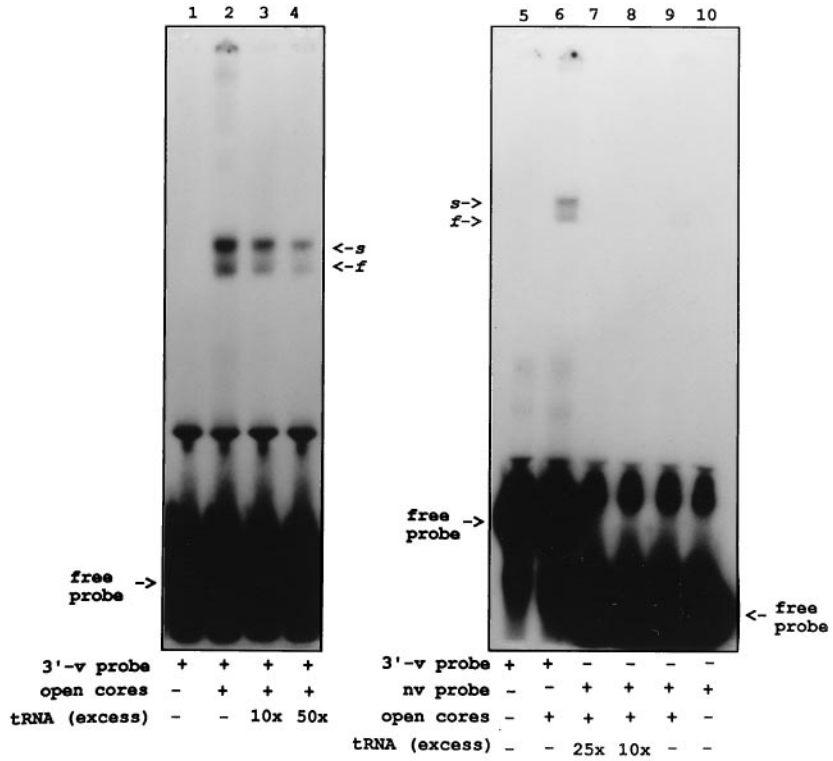

FIG. 2. Specificity of the RNA-binding activity of the open core proteins. ^{32}P-labeled 3′-v probe was used at 0.16 μg (lanes 1 to 4) or 0.25 μg (lanes 5 and 6), ^{32}P-labeled nv probe was used at 0.29 μg, and yeast tRNA was used at 1.6 (lane 3), 8 (lane 4), 2.9 (lane 8), or 14.5 (lane 7) μg. After incubation for 30 min, samples were analyzed for RNA-protein complexes by gel mobility shift assay.

open cores were incubated with ^{32}P-labeled 3′-v RNA for 30 min, after which the reaction mixtures were adjusted to final concentrations of 50, 100, or 150 mM NaCl. Analysis of these mixtures by nondenaturing gel electrophoresis showed that the addition of NaCl did not result in a significant loss of either the *s* or *f* complex (data not shown). Assuming that the overall salt concentration in the cytosol is 100 to 150 mM, this finding implies that the RNA-protein interaction in the *s* and *f* complexes is sufficiently strong for them to be stable under physiological conditions.

Specificity of open core proteins for the 3′ end of viral RNA. Competitive binding assays performed with cold nonrotaviral RNA were used to examine the specificity of the RNA-protein interactions involved in the formation of the *s* and *f* complexes. Open cores were incubated with ^{32}P-labeled 3′-v RNA probe and either a 10- or 50-fold mass excess of yeast tRNA. Under these conditions, the competitor tRNA was present in the reaction mixtures in an approximately 5- or 25-fold molar excess, respectively, over the probe. Analysis of the reaction mixtures by nondenaturing gel electrophoresis showed that the *s* and *f* complexes formed even in the presence of high concentrations of tRNA, albeit at somewhat reduced levels (Fig. 2). More precisely, quantitation revealed a reduction in the formation of the *s* and *f* complexes of approximately 2-fold in the presence of a 10-fold mass (5-fold molar) excess of tRNA and 5-fold in the presence of a 50-fold mass (25-fold molar) excess of tRNA. Similar results were also obtained when brome mosaic virus RNA was used in place of tRNA in the competitive binding assays (data not shown). For example, the addition of a twofold mass excess of brome mosaic virus RNA to reaction mixtures containing open cores and the ^{32}P-labeled 3′-v probe did not interfere with the formation of the *s* and *f* RNA-protein complexes (Fig. 1, lane 3).

To further examine the specificity of the RNA-protein interaction involved in formation of the *s* and *f* complexes, the ^{32}P-labeled RNA probes 5′-v and nv were prepared. The sequence of the 5′-v probe is the same as that of the first 48 nucleotides of gene 8 mRNA, while the sequence of the nv probe is also 48 nucleotides in length but is derived from transcription of SP72-specific sequences. When incubated with open cores, these probes interacted with protein to form complexes that comigrated on nondenaturing gels with or slightly ahead of the *f* complex formed with the 3′-v probe (Fig. 2, lane 9, and Fig. 3, lane 5). The levels of the *f*-like complex formed with the ^{32}P-labeled 5′-v and nv probes were variable between experiments and, in some cases, quite low (Fig. 2, lane 9). Notably, the interaction of core protein and the 5′-v and nv probes never resulted in the formation of an *s*-like complex.

The specificity of the interaction between the core proteins and the 5′-v and nv probes was examined by competitive binding assay. As shown in Fig. 2, lanes 7 to 9, and Fig. 3, lanes 3 to 5, when either a 10- or 25-fold mass (5- or 12.5-fold molar, respectively) excess of tRNA was added to reaction mixtures containing open cores and ^{32}P-labeled 5′-v or nv probe, the *f*-like complex could no longer be detected in the reaction mixtures by mobility shift assay. These findings indicate that, in contrast to the specific interaction between core protein and the 3′-v probe, the interaction between core protein and the 5′-v and nv probes is nonspecific.

VP1 comigrates with the retarded 3′-v probe. To identify the protein component of the *s* and *f* complexes, ^{35}S-labeled open cores were incubated with ^{32}P-labeled 3′-v probe and the re-

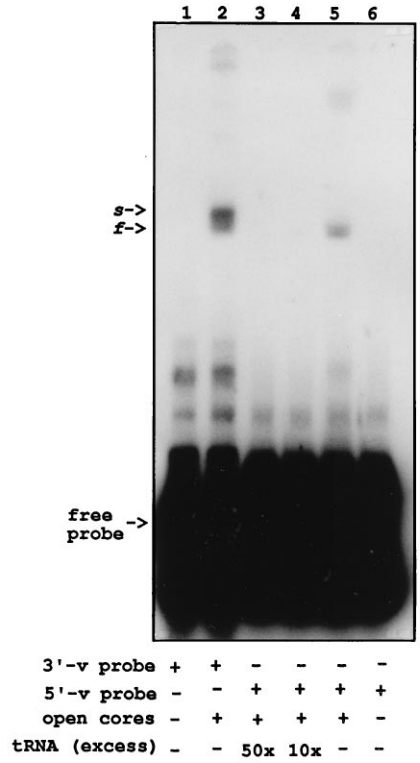

FIG. 3. Gel mobility shift assays with gene 8 5′-terminal probes. ^{32}P-labeled 3′-v probe was used at 0.25 μg, ^{32}P-labeled 5′-v probe was used at 0.15 μg, and yeast tRNA was used at 1.5 (lane 4) or 7.5 (lane 3) μg. After incubation for 30 min, samples were analyzed for RNA-protein complexes by gel mobility shift assay.

action mixture was resolved by nondenaturing gel electrophoresis. ^{35}S-labeled protein in regions of the gel corresponding to the *s* and *f* complexes was recovered and resolved by electrophoresis on an SDS-polyacrylamide gel. The only viral protein identified in the *s* and *f* complexes was VP1, even upon long-term exposure of the gel to film (Fig. 4A). These results indicate that it is the minor core protein VP1 that specifically interacts with the 3′-v probe to form the *f* and *s* complexes. Moreover, these data show that the last 43 nucleotides of gene 8 mRNA contain a specific recognition signal for VP1.

When open cores were incubated in the absence of any probe and the reaction mixture was analyzed by mobility shift assay, no VP1 was detected in regions of the gel where the *s* and *f* complexes would be expected to migrate (Fig. 4B). This finding implied that migration of VP1 into the nondenaturing gel required the protein to interact with the 3′-v probe and that prior to this interaction, VP1 was associated with some other component in the open core preparation which prevented or altered its migration in the gel. To further test this prediction, ^{35}S-labeled open cores were incubated with and without unlabeled 3′-v probe and the reaction mixtures were analyzed by gel mobility shift assay (Fig. 5). Consistent with the prediction, the presence of cold 3′-v probe altered the distribution of ^{35}S-labeled protein in the gel such that bands, although faint, were detectable by autoradiography (Fig. 5, lane 2); these bands were analogous to the *s*- and *f*-complex bands seen when ^{32}P-labeled 3′-v probe was included in reaction mixtures (lane 1). When no probe was present, no ^{35}S-labeled *s*- and *f*-complex bands were detected by the mobility shift assay, presumably because the 3′-v probe was not available to catalyze the dissociation of VP1 from another component in the open core preparation (Fig. 5, lane 3).

RNA-binding and replicase activities associated with core components. In order to obtain additional information about the RNA-binding activity of the core protein components, ^{35}S-labeled open cores were incubated with unlabeled 3′-v probe and then centrifuged on a 10 to 30% glycerol gradient. Fractions collected from the gradient were analyzed by SDS-polyacrylamide gel electrophoresis for the presence of radiolabeled protein. As shown in Fig. 6A, centrifugation resulted in the separation of the core protein components into three distinct populations, one that contained VP1 and no VP2 or VP3 (fractions 6 to 10), another that contained VP1 and VP3 and a small amount of VP6 (fractions 11 to 14), and another that consisted predominantly of VP2 but also included small amounts of VP1 and VP6 (fractions 23 to 25 and the pellet). The fractions containing predominantly VP1 (fractions 6 to 10) and VP1 and VP3 (fractions 11 to 14) also contained trace amounts of a protein with a molecular size between that of VP1 (125 kDa) and VP2 (102 kDa) (Fig. 6A). The origin of this protein is unknown, but perhaps this protein is a cleavage product of VP1. Because of its significantly higher sedimentation rate compared with that of the larger protein VP1 (fractions 6 to 8), VP3 (98 kDa) detected in fractions 11 to 14 may exist as a complex with VP1 or may be multimeric. The rapid rate of sedimentation of VP2 in the glycerol gradient is consistent with the protein being a component of open cores. In fact, electron microscopic analysis of the open core preparation and the pellet fraction of the glycerol gradient confirmed

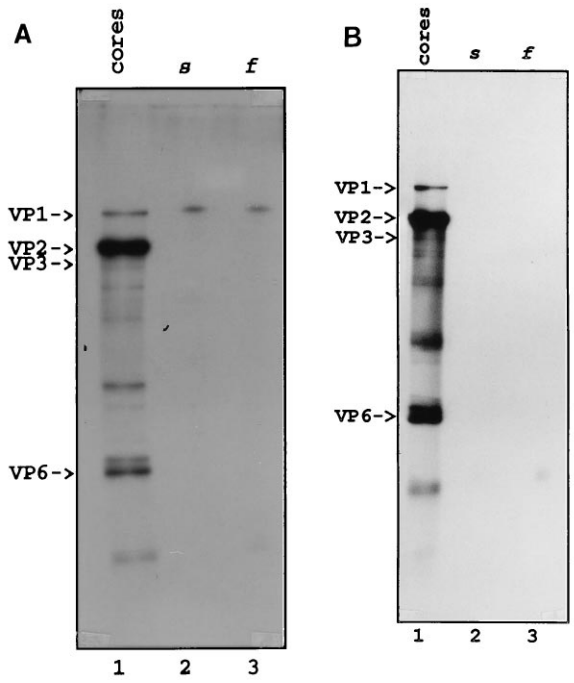

FIG. 4. Detection of VP1 in RNA-protein complexes. ^{35}S-labeled open cores were incubated with the ^{32}P-labeled 3′-v probe (A) or with no probe (B). The samples were then resolved by electrophoresis on nondenaturing polyacrylamide gels. Regions of the gel corresponding to the *s* and *f* complexes were recovered and rehydrated in Laemmli sample buffer. The ^{35}S-labeled protein components of the gel pieces were detected by electrophoresis on SDS–12% polyacrylamide gels and by fluorography. As a protein marker, an aliquot of the preparation of ^{35}S-labeled open cores was electrophoresed in parallel lanes. The open core preparation contains residual VP6 and minor bands migrating between full-length VP2 and VP6 that are presumed to represent degradation products of VP2 (30).

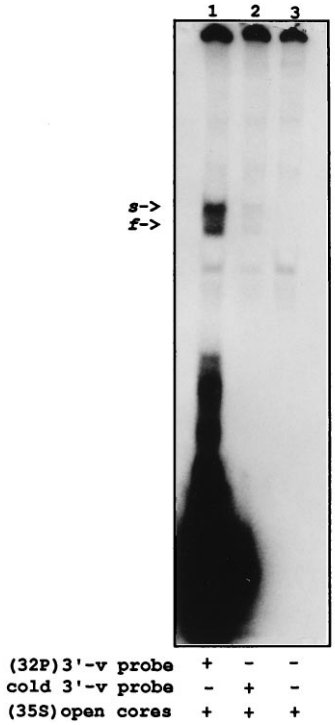

FIG. 5. Effect of the gene 8 3′-terminal probe on the migration of ^{35}S-labeled protein on a nondenaturing gel. ^{35}S-labeled open cores were incubated with ^{32}P-labeled 3′-v probe (lane 1), cold 3′-v probe (lane 2), or no probe (lane 3). The samples were analyzed by electrophoresis on a nondenaturing 8% polyacrylamide gel and by autoradiography.

the presence of core-like structures (data not shown). Proteins in the pellet and fractions 20 to 25 that are intermediate in size between full-length VP2 and VP6 probably are VP2 cleavage products (Fig. 6A) (30).

The RNA-binding activity of core proteins resolved by centrifugation was examined by incubating portions of gradient fractions with the ^{32}P-labeled 3′-v probe and then analyzing the reaction mixtures for the formation of RNA-protein complexes by gel mobility shift assay. The results showed that the *s* and *f* RNA-protein complexes were formed readily and nearly exclusively by fractions containing predominantly VP1 (fractions 6 to 8 [Fig. 6B]). Therefore, consistent with data presented above (Fig. 4A), VP1 appears to be able to specifically recognize and bind to a sequence element at the 3′ end of gene 8 mRNA in the absence of any other viral protein.

In contrast to fractions containing predominantly VP1, incubation of the VP1-VP3-containing fractions 11 to 13 with the 3′-v probe did not give rise to the *s* and *f* complexes. Instead, the RNA-binding activity of proteins in these fractions gave rise to a novel RNA-protein complex designated *h* (Fig. 6B). The identity of the proteins involved in the formation of the *h* complex is not clear, but these proteins may include VP1. Otherwise, the VP1 in the VP1-VP3-containing fractions would have been expected to behave similarly to the VP1 in fractions 6 to 8 and would have led to the formation of the *s* and *f* complexes. The fact that the *h* RNA-protein complex migrated only slightly into the gel suggests that the protein component of the complex is large and therefore multimeric in nature and may contain both VP1 and VP3 (Fig. 6B). Studies to further define the specificity and composition of the *h* RNA-protein complex are under way. A correlation was noted between the amount of VP2 contained in the gradient fractions (Fig. 6A) and the amount of probe retained in the wells of the nondenaturing gels when those fractions were assayed for RNA-binding activity (Fig. 6B). This phenomenon probably stems from the fact that as a component of open cores, VP2 is associated with complexes that are too large to enter the nondenaturing 8% polyacrylamide gels used in the mobility shift assays. Given that VP2 has nonspecific RNA-binding activity (11), the retention of the protein in the well probably in turn causes the retention of some of the probe.

To examine the relationship between the assembly of RNA-protein complexes and the formation of complexes with replicase activity, portions of the gradient fractions were assayed for replicase activity by using the system developed by Chen et al. (4). Reaction mixtures contained gene 8 mRNA as the template for the synthesis of dsRNA and [^{32}P]UTP to label newly synthesized RNA. Analysis of the reaction products for ^{32}P-labeled gene 8 dsRNA indicated that only the gradient pellet and the fraction adjacent to the pellet (fraction 25) possessed replicase activity (Fig. 6C). Even upon exposure of the gel to film for extended periods of time, no ^{32}P-labeled dsRNA was detected in the VP1-containing fractions 6 to 8 or the VP1-VP3-containing fractions 11 to 13. These findings indicate that although VP1 can specifically interact with the 3′ end of viral mRNA, this interaction in itself is not sufficient to produce an RNA-protein complex with replicase activity. Likewise, the combination of VP1 and VP3 is not sufficient to support the formation of an RNA-protein complex that contains replicase activity (Fig. 6C). Instead, because the only portions of the gradient with replicase activity were also the only portions containing high levels of VP2, it is likely that VP2 is an essential component of complexes with replicase activity. However, because VP2 CLPs alone lack replicase activity (31), another protein in the portions of the gradient with replicase activity, presumably VP1, also is required for minus-strand synthesis.

VP1 stimulates replicase activity. As an approach for further testing the role of VP1 in replication, increasing portions of a gradient fraction containing predominantly VP1 (fraction 6 [Fig. 6A]) were combined with a fixed amount of the VP2-rich pellet and the mixture was assayed for replicase activity. Electrophoretic analysis showed that while VP1 alone lacked replicase activity, the addition of increasing amounts of the purified VP1 to the pellet significantly increased the level of gene 8 dsRNA synthesis (Fig. 7). The results were quantitated by densitometry, and the stimulation in dsRNA synthesis per unit of added VP1 was calculated (Table 1). The analysis revealed that the addition of 5, 10, and 18 μl of VP1 stimulated gene 8 dsRNA replication 3.9-, 5.2-, and 6.5-fold, respectively. When the replication levels were adjusted to a constant amount of

TABLE 1. Stimulation of replication by gradient-purified VP1

Vol (μl) of added VP1[a]	Relative level[b] of:		Stimulation in dsRNA synthesis/constant amt of added VP1[c]
	dsRNA product	Total VP1	
0	1	1	
5	3.9	1.27	14.4
10	5.2	1.54	9.6
18	6.5	1.97	6.7

[a] Volume of purified VP1 (fraction 6) added to reaction mixtures as described in the legend to Fig. 7.

[b] The integrated intensity of bands detected on polyacrylamide gels was determined by densitometry and normalized to a value of 1 for the reaction mixture containing no added VP1.

[c] Value obtained by dividing the relative level of dsRNA product by the relative level of added VP1. The relative level of added VP1 is defined as the relative level of total VP1 minus 1.

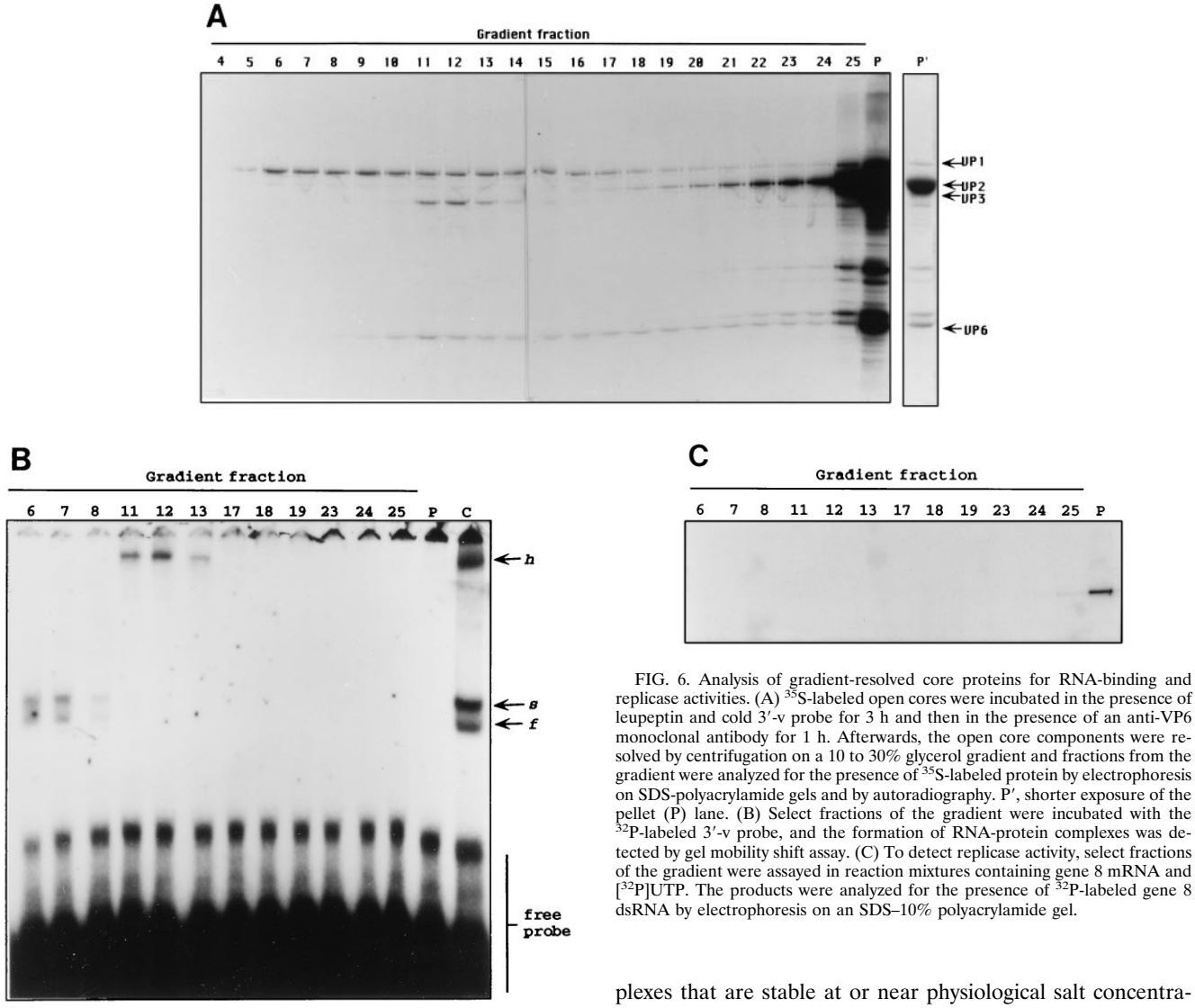

FIG. 6. Analysis of gradient-resolved core proteins for RNA-binding and replicase activities. (A) ^{35}S-labeled open cores were incubated in the presence of leupeptin and cold 3′-v probe for 3 h and then in the presence of an anti-VP6 monoclonal antibody for 1 h. Afterwards, the open core components were resolved by centrifugation on a 10 to 30% glycerol gradient and fractions from the gradient were analyzed for the presence of ^{35}S-labeled protein by electrophoresis on SDS-polyacrylamide gels and by autoradiography. P′, shorter exposure of the pellet (P) lane. (B) Select fractions of the gradient were incubated with the ^{32}P-labeled 3′-v probe, and the formation of RNA-protein complexes was detected by gel mobility shift assay. (C) To detect replicase activity, select fractions of the gradient were assayed in reaction mixtures containing gene 8 mRNA and [^{32}P]UTP. The products were analyzed for the presence of ^{32}P-labeled gene 8 dsRNA by electrophoresis on an SDS–10% polyacrylamide gel.

added VP1, the addition of small amounts of VP1 were determined to stimulate dsRNA synthesis to a greater extent than the addition of larger amounts of VP1 (Table 1). This may indicate that at higher concentrations of added VP1, VP1 is in excess and that other components, such as VP2 or gene 8 mRNA, have become limiting factors in the reaction mixtures. In sum, these data are consistent with the hypothesis that VP1 can specifically bind to viral mRNA in the absence of other viral proteins but that this complex requires the presence of VP2 for replicase activity.

DISCUSSION

The synthesis of rotavirus minus-strand RNA requires that at least one of the core proteins recognize an essential *cis*-acting replication signal located near the 3′ end of each viral mRNA. To identify the protein(s) that interacts with the 3′ *cis*-acting signal, a gel mobility shift assay was developed in order to study the interaction of open core components with virus- and non-virus-specific RNAs. The results showed that the minor core protein VP1 specifically interacts with a signal at the 3′ end of gene 8 mRNA to form RNA-protein complexes that are stable at or near physiological salt concentrations. Moreover, assay of gradient-purified VP1 indicated that the formation of the RNA-protein complexes does not require the participation of other viral proteins. Previous studies have shown that VP1 binds nucleotides (26), contains sequence motifs shared by RNA polymerases (6, 7, 18), and is a common minor component of complexes with replicase activity (8). These properties and the discovery that VP1 recognizes the 3′ end of viral mRNA are consistent with VP1 functioning as the viral RNA polymerase. However, while the protein may hypothetically serve as the viral polymerase, our results demonstrated that gradient-purified VP1 alone lacks polymerase activity, as determined by assay in the cell-free replication system. But when VP1 was added to VP2-containing samples in the cell-free replication system, replicase activity was stimulated severalfold. These findings are consistent with the hypothesis that VP1 functions as the viral replicase but that VP2 is essential for its activity. Evidence that VP2 is required for RNA replication was obtained in earlier studies examining the enzymatic properties of replication intermediates formed in cells infected with rotavirus temperature-sensitive mutants (15). Indeed, results from these studies suggest that it is the assembly of VP2 into cores that may be essential for the formation of complexes with replicase activity.

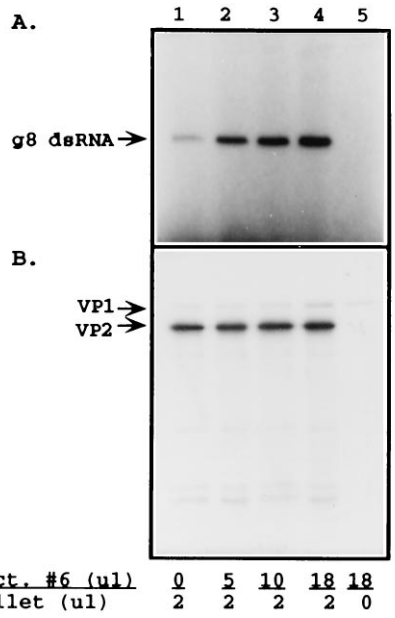

FIG. 7. Effect of gradient-purified VP1 on replicase activity. (A) Portions of fraction (fract.) 6 and the pellet of the gradient described for Fig. 6A were combined and assayed for replicase activity in reaction mixtures containing gene 8 mRNA and [^{32}P]UTP. ^{32}P-labeled gene 8 dsRNA synthesized in the samples was detected by electrophoresis of the samples on an SDS–10% polyacrylamide gel. (B) ^{35}S-labeled protein added to the replicase assays was analyzed by electrophoresis on a SDS–12% polyacrylamide gel.

In a recent study by Zeng et al. on the role of the core proteins in minus-strand synthesis, recombinant baculoviruses were used to produce VP1-VP2 and VP2 CLPs (31). By analysis of the particles in a cell-free replication system, the VP1-VP2 CLPs were shown to have replicase activity whereas the VP2 CLPs were shown to lack such activity. While these results indicated that VP1 is essential for minus-strand synthesis and VP2 alone lacks replicase activity, the study by Zeng et al. did not show whether VP1 alone possessed replicase activity or whether VP2 was an essential component of complexes with replicase activity. Our results are the first to directly demonstrate that VP1 alone lacks replicase activity. Previous studies have shown that the only essential *cis*-acting signal required for minus-strand synthesis in vitro is formed by the last seven nucleotides of rotavirus mRNA (21, 27). Given that of the three core proteins, only VP1 specifically interacts with the 3′ end of viral mRNA, it is likely that VP1 recognizes and binds to the *cis*-acting replication signal. However, this remains to be directly tested. VP3 can be ruled out as the core protein that recognizes the 3′ essential replication signal since Zeng et al. showed that VP1-VP2 CLPs retain replicase activity despite the absence of VP3 (31). And scrutiny of the RNA-binding activity of VP2 has indicated that its affinity for RNA is nonspecific (11).

The formation of empty VP1-VP2 CLPs in baculovirus expression systems shows that protein-protein interactions between VP1 and the VP2 shell of the core occur (31). Moreover, as the viral polymerase, VP1 can be presumed to interact with the dsRNA genome within the core. Inspection of cores incubated in LSB by electron microscopy indicates that this treatment causes the partial disruption of the VP2 shell and the loss of the dsRNA genome (4). The fate of VP1 in cores treated with LSB is not known, but the VP1 may remain associated with the VP2 shell or with the dsRNA released from the core. Our studies indicate that VP1 is associated with some other component of the open core preparation and that the presence of viral RNA leads to the destabilization of this association and the formation of complexes between the released VP1 and the RNA. VP1 may interact with viral mRNA in more than one way, given that upon gel mobility shift assay, two closely migrating complexes, designated *s* and *f*, were formed by VP1 and the gene 8 3′-terminal probe, 3′-v. Exactly how the *s* and *f* complexes differ is unclear, but perhaps their VP1 components differ with respect to conformation.

Interestingly, not all the VP1 that was resolved by centrifugation of open cores on glycerol gradients interacted with the 3′-v RNA to form the *s* and *f* complexes. Specifically, the RNA-binding activity of gradient fractions containing VP1 and VP3 gave rise to the *h* complex but not the *s* or *f* complex. This observation indicates that the form of VP1 in the VP1-VP3-containing fractions is somehow different from the form in the fractions containing VP1 alone. Perhaps VP1 and VP3 exist as a heteromultimer in the VP1-VP3-containing fractions and it is this structure that interacts with the 3′-v RNA to form the *h* complex. As the putative viral guanylyltransferase, VP3 is likely to have affinity for the 5′ end of viral plus-strand RNA, i.e., mRNA, and therefore a VP1-VP3 complex may play a role in initiating transcription of the dsRNA genome segments.

Of the 11 proteins encoded by the rotavirus genome, 5 (VP1, VP2, NSP1, NSP2, and NSP3) have demonstrated RNA-binding activity and 2 (VP3 and NSP5) have suspected RNA-binding activity (20). VP1 is the second protein shown to specifically recognize the 3′ end of rotavirus mRNA. The first, the nonstructural protein NSP3, recognizes the last 4 to 5 nucleotides of viral mRNA (23, 24), the same nucleotides that make up part of the 3′ essential replication signal (21). What effect the binding of NSP3 to the 3′ end of viral mRNA has on the binding of VP1 to the mRNA and on the synthesis of minus-strand RNA has yet to be determined. The analysis of the RNA-binding activity of VP1 indicates that it is this protein which has primary responsibility for selecting viral mRNAs that are to be replicated and packaged into progeny cores. How the 11 mRNAs are distinguished during the replication and packaging process such that the progeny cores contain the complete complement of 11 dsRNAs remains an intriguing mystery.

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