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Rotavirus RNA Polymerase Requires the Core Shell Protein To Synthesize the Double-Stranded RNA Genome

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Rotavirus cores contain the double-stranded RNA (dsRNA) genome, RNA polymerase VP1, and guanylyltransferase VP3 and are enclosed within a lattice formed by the RNA-binding protein VP2. Analysis of baculovirus-expressed core-like particles (CLPs) has shown that VP1 and VP2 assemble into the simplest core-like structures with replicase activity and that VP1, but not VP3, is essential for replicase activity. To further define the role of VP1 and VP2 in the synthesis of dsRNA from viral mRNA, recombinant baculoviruses containing gene 1 (rBVg1) and gene 2 (rBVg2) of SA11 rotavirus were generated and used to express recombinant VP1 (rVP1) and rVP2, respectively. After purification, the proteins were assayed individually and together for the ability to catalyze the synthesis of dsRNA in a cell-free replication system. The results showed that dsRNA was synthesized only in assays containing rVP1 and rVP2, thus establishing that both proteins are essential for replicase activity. Even in assays containing a primer-linked mRNA template, neither rVP1 nor rVP2 alone directed RNA synthesis. Characterization of the *cis*-acting replication signals in mRNA recognized by the replicase of rVP1 and rVP2 showed that they were the same as those recognized by the replicase of virion-derived cores, thus excluding a role for VP3 in recognition of the mRNA template by the replicase. Analysis of RNA-protein interactions indicated that the mRNA template binds strongly to VP2 in replicase assays but that the majority of the dsRNA product neither is packaged nor stably associates with VP2. The results of replicase assays performed with mutant VP2 containing a deletion in its RNA-binding domain suggests that the essential role for VP2 in replication is linked to the protein's ability to bind the mRNA template for minus-strand synthesis.

Rotaviruses, members of the family *Reoviridae*, are an important cause of severe dehydrating diarrhea in infants and young children (3, 16). The rotavirus consists of three concentric layers (shells) of protein and 11 segments of double-stranded RNA (dsRNA) (34, 48). The outer shell contains the glycoprotein VP7 and the trypsin-activated spike protein VP4, while the intermediate shell is formed by VP6 trimers (9). The inner most shell is a T=1 icosahedron consisting of 60 dimers of VP2 (9, 40), a protein with nonspecific RNA-binding activity (20). One copy each of VP1 and VP3 have been proposed to exist at each of the 12 vertices of the innermost shell (23, 35). Collectively, VP1, VP2, VP3, and the genome form the core of the virion. Cores disrupted by incubation in hypotonic buffer (open cores) have an associated replicase activity which catalyzes the synthesis of dsRNA from viral mRNA in vitro (5). Viral transcriptase activity is associated with cores surrounded by VP6, i.e., double-layered particles, and is responsible for the synthesis of viral mRNA (2, 7, 38).

Rotavirus mRNAs are unique in that they possess 5′ cap structures but lack 3′ poly(A) tails (15, 27). Cell-free replication systems containing open cores have been used to locate *cis*-acting signals in the mRNAs that promote minus-strand synthesis to form dsRNA (31, 46, 47). Only one of the three signals identified so far, that which is located at the extreme 3′ end of viral mRNAs, was shown to be essential for the synthesis of dsRNA. The other two signals, while not essential, were found to enhance the synthesis of dsRNA. These enhancement signals were mapped to the 5′ untranslated region (UTR) and to the 5′ end of the 3′ UTR of the viral mRNA.

The ability of open cores to direct the synthesis of dsRNA indicates that one or more of the core proteins are essential components of the viral replicase. By analysis of baculovirus-expressed core-like particles (CLPs), Zeng et al. (49) showed that VP1-VP2 CLPs have associated replicase activity but that VP2 and VP2-VP3 CLPs do not. These findings established that VP1 is essential for replicase activity but that the viral guanylyltransferase, VP3 (24, 33), is not. The known properties of VP1 are consistent with the protein functioning as an RNA polymerase: (i) VP1 contains sequence motifs that are common to RNA-dependent RNA polymerases (8, 11, 28); (ii) VP1 binds nucleotides, and cross-linking of the photoreactable nucleotide analog azido-ATP to VP1 inhibits RNA polymerase activity (44); (iii) VP1 specifically recognizes the 3′ end of viral mRNA (29); and (iv) VP1 is a common component of baculovirus-expressed particles and replication intermediates that have replicase activity (12, 49). Because baculovirus-expressed VP1 was not assayed alone for replicase activity, Zeng and colleagues (49) were not able to determine whether this protein alone or only when in combination with VP2 catalyzes dsRNA synthesis. Indeed, VP2 may be essential for replicase activity given that replication intermediates with replicase activity are not formed at a nonpermissive temperature in cells infected with *ts*F, a mutant rotavirus with a temperature-sensitive (*ts*) lesion that maps to the VP2 gene (25).

To identify the minimal essential components of the viral replicase, we have individually expressed and purified recombinant VP1 (rVP1) and rVP2 and then assayed these proteins

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separately and in combination for associated replicase activity. The results demonstrated that replicase activity is dependent on the presence of both VP1 and VP2 and hence point to a mechanism for coordinating rotavirus RNA replication with the assembly of virion cores. We obtained evidence indicating that the essential role for VP2 in replication is linked to the protein's ability to bind the mRNA template for minus-strand synthesis.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* (Sf9) cells were grown in TNM-FH medium containing 10% fetal bovine serum (42). Simian SA11 and bovine UK rotaviruses were propagated in MA014 cells, and virions were purified by CsCl centrifugation (31). Double-shelled particles were recovered from EDTA-treated virions by CsCl centrifugation (31). Open cores were prepared as described earlier from SA11-4F virions (5).

Preparation of recombinant baculoviruses (rBVs). To construct plasmid pBS-SKg1, SA11 double-shelled particles were used to synthesize viral mRNA in vitro (6). A gene 1 cDNA was made by incubating the mRNA with Moloney murine leukemia virus reverse transcriptase and the minus-sense primer, g1-3′sph (5′-ctagaactagTCTAATCTTGAAAGAAGT-3′). The gene 1 cDNA was amplified in a PCR containing *Taq* DNA polymerase, the plus-sense primer, g1-5′sph (5′-gatcccactagtATGGGGAAGTACAATCTA-3′), and g1-3′sph (6). Sequences in the primers that are virus specific are shown in uppercase, and those that generated *Sph*I restriction sites in the gene 1 cDNA are underlined. The amplified gene 1 cDNA was digested with *Sph*I, gel purified, and ligated into the vector pBluescript SK which had been linearized with *Sph*I and treated with alkaline phosphatase (37). pBS-SKg1 contains the complete open reading frame for VP1 but lacks the complete 5′ and 3′ UTRs of authentic gene 1 message. Plasmid pT7g2SA11-R contains a full-length cDNA of the gene (segment 2) encoding VP2; its construction and sequence have been reported previously (26).

Rotavirus-specific cDNAs synthesized by PCR were inserted into the baculovirus transfer vector pCR-Bac according to the protocols of the supplier (Invitrogen Corp.). The primers used to prepare cDNAs encoding full-length VP1 were g1-5′-BV (5′-AGCATGGGGAAGTACAATCTAATCTTG-3′) and g1-3′-BV (5′-GAAGAAAGTTCTAATCTTGCGAAT-3′), and those encoding VP2 were g2-5′-BV (5′-AGCATGGCGTATCGAAAACGTGGA-3′) and g2-3′-BV (5′-GCGTTTACAGTTCGTTCATGAT-3′). The primers used to prepare a cDNA encoding VP2 with an NH_2-terminal deletion of 25 amino acids were g2-5′-delN1-25 (5′-ATGAGCAAGAAGATTAATAATGACAGTCCTAA A-3′) and g2-3′-BV. The locations of start codons in the 5′ primers are underlined. Directed insertion of cDNAs into the transfer vector was achieved by phosphorylation of the forward primers g1-5′-BV, g2-5′-BV, and g2-5′-delN1-25. pBS-SKg1 and pT7g2SA11-R were included in amplification reactions containing *Taq* polymerase as templates for the synthesis of gene 1 and gene 2 cDNAs, respectively. To produce rBVs expressing full-length VP1 (rBVg1-11), full-length VP2 (rBVg2-15), and NH_2-truncated VP2 (rBVg2d1-132), the transfer vectors pCR-Bacg1-4, pCR-Bacg2-18, and pCR-Bacg2del1-132 (B2-17), respectively, were transfected with Bac-N-Blue linear *Autographa californica* nuclear polyhedrosis virus DNA (Invitrogen) into Sf9 cells. The rBVs were selected and plaque purified on the basis of the ability to form blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and to express protein of the expected size. The nucleotide sequence of the gene 1 open reading frame in rBVg1-11 was determined by dideoxynucleotide sequencing (39).

Purification of recombinant VP1 and VP2. Sf9 cells were washed with phosphate-buffered saline, infected with 3 PFU of rBVg1, rBVg2, or rBVg2d1-132 per cell, and maintained as spinner cultures in TNM-FH medium containing 2.5% fetal bovine serum and 0.25 μg each of the protease inhibitors leupeptin and aprotinin per ml. After 7 days, the rBVg1-infected cells were recovered by low-speed centrifugation, washed three times with cold phosphate-buffered saline, resuspended in cold LSB (2 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 0.5 mM dithiothreitol), and incubated on ice for 5 min. The cell suspension was adjusted to 0.1% deoxycholate (DOC), gently mixed until the suspension cleared, and then incubated on ice for 2 min. The sample was centrifuged at low speed, and the pellet was resuspended in LSB–0.1% DOC. The suspension was dialyzed exhaustively against LSB and used as the source of rVP1 in replicase assays. At 4 days postinfection, rBVg2- and rBVg2d1-132-infected cells were lysed by suspension in 10 mM Tris-HCl (pH 7.1)–1% DOC–1 mM EDTA–1 μg each of leupeptin and aprotinin per ml. Each lysate was sonicated, and the recombinant protein was pelleted through a 5-ml cushion of 35% (wt/vol) sucrose in Tris-buffered saline by centrifugation for 90 min at 25,000 rpm in a Beckman SW27.1 rotor. Pellets were suspended in Tris-buffered saline and sonicated, and the samples were adjusted to 1.31 g of CsCl per cm^3 and centrifuged for 18 h at 33,000 rpm in a Beckman SW50.1 rotor. Recombinant protein banding at 1.30 g/cm^3 was collected and dialyzed exhaustively against LSB and used as the source of rVP2 or rVP2d1-132 in replicase assays. The rVP2 preparation was spread on carbon-Formvar-coated grids, stained with 3% phosphotungstic acid, and examined for CLPs by electron microscopy. The concentration of total protein in the rVP1 and rVP2 preparations was determined with a Bio-Rad protein assay kit. The concentration of rVP1, rVP2, and rVP2d1-132 in preparations was determined by coelectrophoresis of samples of the recombinant proteins with known amounts of bovine serum albumin on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Novex, San Diego, Calif.), staining the gels with Coomassie brilliant blue R-250, and then comparing the intensities of the bands of rVP1, rVP2, and rVP2d1-132 with those of bovine serum albumin.

Construction of templates for T7 transcription. SP65g8R and the pCITEg8R vectors were used to produce wild-type gene 8 mRNA and the pCITEg8 -4, -6, -7, -10, -20 and -40 RNAs, respectively, and were constructed as described elsewhere (31). Wild-type gene 6 mRNA was synthesized by runoff transcription of plasmid pMJ5B6.4. To construct the plasmid, mRNA was synthesized in vitro from double-shelled particles of bovine UK rotavirus (6). A gene 6 cDNA was prepared from the mRNA in a reaction mixture containing Moloney murine leukemia virus reverse transcriptase and the negative-sense primer UK6.2 (5′-CCGCGGACTCATCCTCTCACTACGCG-3′). An appropriate sequence was included in the UK6.2 primer (underlined) to generate a *Sac*II site in pMJ5B6.4 immediately downstream of the gene 6 cDNA. The cDNA was amplified with *Taq* polymerase, the positive-sense primer UK6.1 (5′-CTTTTAAACGAAGTC TTC-3′), and UK6.2. The amplified gene 6 cDNA product was phosphorylated, gel purified, and then ligated into the *Stu*I site of the vector pMJ5. Prior to transcription, constructs were linearized with *Sac*II and treated with T4 DNA polymerase (31). The expected nucleotide sequences of the constructs were verified by dideoxynucleotide sequencing (39).

In some cases, gene 8 RNAs were synthesized from PCR products made by amplification with *Taq* polymerase. The PCR products used as templates for the synthesis of the wild-type gene 8 mRNA, the 5′-modified RNA g8R5′d1-50, and the 3′-modified RNAs g8R3′(-9)-(-41) and g8R3′(-9)-(-66) were prepared as described before (31). The PCR product that was used to synthesize a gene 8 mRNA containing a 3′-terminal hairpin (gene 8HP RNA) was produced in an amplification reaction containing the positive-sense primer T7g8R+ and the negative-sense primer g8hp (5′-AGCGCTTATGTGACCGGTCACATAAGC GCTTTCTATTCTTGCTAAGCCATC-3′), *Taq* DNA polymerase (Elongase; Life Sciences), and SP65g8R as the template (6). The underlined sequence generates the 15-base 3′ extension of the gene 8HP RNA predicted to base pair with the last 15 bases to form a hairpin structure. Prior to transcription, templates produced by PCR were treated with T4 DNA polymerase to remove 5′-overhang A residues.

Synthesis of mRNAs. Unlabeled RNAs were synthesized by runoff transcription with Ambion MEGAscript T7 transcription kits (31). ^{32}P-labeled gene 8 mRNA was synthesized in reaction mixtures that contained 0.75 mM UTP and 50 μCi of [^{32}P]UTP (800 Ci/mmol).

Cell-free replication system. The reaction conditions used initially to test rVP1 and rVP2 for replicase activity were like those used previously to analyze the replicase activity of open cores (5, 31) and henceforth are referred to as the unmodified conditions. Replicase assays performed under the unmodified conditions contained 50 mM Tris-HCl (pH 7.1), 10 mM magnesium acetate, 1.5% polyethylene glycol, 1.5 mM dithiothreitol, 1.5 U of RNasin, 1.25 mM each ATP, CTP, GTP, and UTP, 750 μCi of [^{32}P]UTP (800 Ci/mmol) per ml, and 50 to 100 μg of each template mRNA per ml. Based on the results of assays optimizing the replicase activity associated with rVP1 and rVP2, the reaction mixtures were altered (modified conditions) to include 25 mM $MgCl_2$ and approximately 25 μg of rVP1 and 200 μg of rVP2 per ml. Reaction mixtures were incubated for 4 to 6 h at 32°C. Afterwards, 2.5 μl of proteinase K (10 mg/ml) and 100 μl of Laemmli sample buffer (21) were added, and the reaction mixtures were incubated for 15 min at 37°C. ^{32}P-labeled dsRNA in the samples was detected by polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels containing 0.5% SDS and by autoradiography (21). Bands of ^{32}P-labeled dsRNA were quantified with a Molecular Dynamics PhosphorImager (model 445 SI).

Detection of RNA-protein interactions. To test for binding of the mRNA to rVP2 in the cell-free replication system, 10 μg of ^{32}P-labeled gene 8 mRNA was incubated with 10 μg of rVP2 in a 200-μl reaction mixture lacking rVP1 and [^{32}P]UTP. After addition of 200 μl of TMN buffer (3 mM Tris-HCl [pH 8.1], 3 mM $MgCl_2$, 66 mM NH_4Cl, 14 mM potassium acetate, 1 mM dithioerythritol), this sample and a control sample containing 10 μg of ^{32}P-labeled gene 8 mRNA in 400 μl of TMN were layered over 4-ml 15 to 30% (wt/vol) sucrose gradients in TMN buffer. Following centrifugation for 1 h at 35,000 rpm in a Beckman SW50.1 rotor at 4°C, 500-μl fractions were collected and the pellets were resuspended in 100 μl of TMN buffer. Portions of each fraction (50 μl) and pellet (10 μl) were assayed for ^{32}P-labeled mRNA by electrophoresis on nondenaturing 8% polyacrylamide gels containing 50 mM Tris-glycine buffer (pH 8.8) for 16 h at 150 V (17). ^{32}P-labeled mRNA was detected in gels by autoradiography.

The possible association of dsRNA products with recombinant proteins in the cell-free replication system was examined by incubating 10 μg of unlabeled gene 8 mRNA with 1.25 μg of rVP1 and 10 μg of rVP2 under the modified reaction conditions. The sample was centrifuged on a 15 to 30% sucrose gradient (as described above), and the gradient fractions and the pellet were analyzed for ^{32}P-labeled dsRNA by electrophoresis on a nondenaturing 8% polyacrylamide gel (17) and on a 10% polyacrylamide gel containing SDS (21) and by autoradiography. Proteins in gradient fractions and the pellet were detected by SDS-PAGE and staining with Coomassie brilliant blue.

Nucleotide sequence accession number. The GenBank accession number for the gene 1 sequence is AF015955.

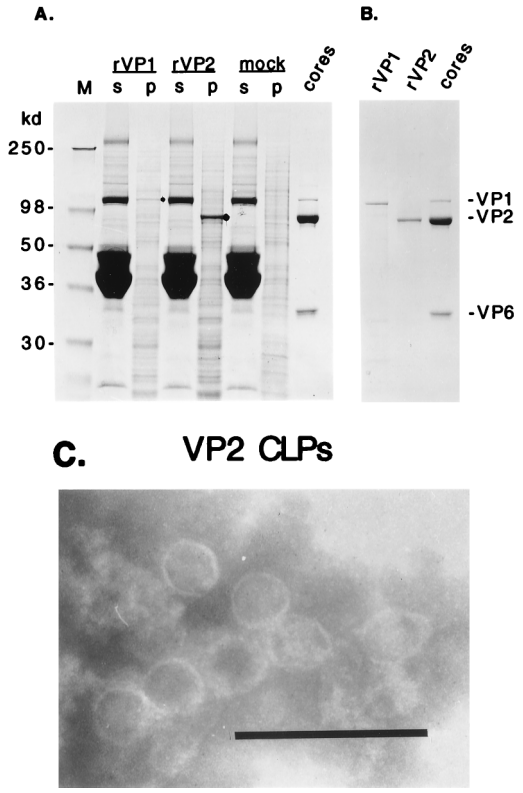

FIG. 1. Expression of rVP1 and rVP2. (A) rBVg1- and rBVg2-infected and mock-infected Sf9 suspension cultures were centrifuged at low speed, and the supernatant(s) and cell pellet (p) were analyzed for rVP1 and rVP2 by SDS-PAGE and by staining with Coomassie blue. Positions of the expressed proteins in the gel are indicated with dots. (B) Preparations of rVP1 and rVP2 recovered from rBVg1- and rBVg2-infected Sf9 cells were similarly analyzed. Cores purified from SA11-4F rotavirus and prestained molecular weight markers (M) were electrophoresed in parallel lanes. (C) Purified rVP2 was stained with phosphotungstic acid and examined directly for the presence of CLPs by electron microscopy. The size bar represents 100 nm.

RESULTS

Expression of rVP1 and rVP2. To assess the importance of VP1 and VP2 in the synthesis of rotavirus dsRNA, cDNAs were prepared to SA11 gene 1 and gene 2, and their nucleotide sequences were determined. The predicted amino acid sequence of VP1 encoded by the gene 1 cDNA showed 99% identity with the sequence published previously for SA11 VP1 (data not shown) and contained the same polymerase motifs that are shared among the putative RNA polymerases of rotaviruses, reoviruses, bluetongue viruses, and phytoreoviruses (4, 43). The predicted amino acid sequence of VP2 encoded by the gene 2 cDNA has been reported elsewhere and is similar to the VP2 sequences of other group A rotaviruses (26). The gene 1 and 2 cDNAs were used to generate rBVs which expressed VP1 (rBVg1) and VP2 (rBVg2), respectively (Fig. 1A). rVP1 was recovered from lysates of rBVg1-infected Sf9 cells by differential centrifugation (Fig. 1B). rVP2 was purified from DOC-treated rBVg2-infected cells by centrifugation on CsCl gradients, where it banded at a density of 1.30 cm^3 (Fig. 1B). Consistent with earlier reports (19), examination of the purified rVP2 by electron microscopy revealed that the protein possessed the ability to self-assemble into approximately 30 nm CLPs (Fig. 1C).

Reconstitution of replicase activity. Purified rVP1 and rVP2 were incubated individually and together with viral gene 6 or 8 mRNA in a cell-free replication system under conditions shown previously to support minus-strand synthesis and the formation of gene 8 dsRNA by open cores (31). The gene 6 and 8 mRNAs included in reaction mixtures contained bona fide 5′ and 3′ termini and were generated by T7 transcription of the plasmids pT7g6 and SP65g8R, respectively. [^{32}P]UTP was included in reaction mixtures to label newly synthesized dsRNA. Analysis of the products by SDS-PAGE indicated that neither rVP1 nor rVP2 alone supported the synthesis of gene 6 or 8 dsRNA (Fig. 2). In contrast, when rVP1 and rVP2 were included in the cell-free system, gene 6 and 8 dsRNAs that were identical in size to the gene 6 and 8 dsRNAs made by reaction mixtures containing open cores instead of recombinant protein were produced. No dsRNA products were made in reaction mixtures that contained rVP1 and rVP2 but lacked template mRNA. Together, these data establish that both VP1 and VP2 are sufficient and essential for minus-strand synthesis (Fig. 2). Analysis of the synthesis of dsRNA in reaction mixtures containing rVP1 and rVP2 and gene 8 mRNA indicated that the replicase remained functional for approximately 5 h (not shown).

To determine the ratio of rVP1 and rVP2 that would stimulate the maximum level of replicase activity in vitro, inversely varying amounts of rVP1 and rVP2 were incubated in reaction mixtures containing gene 8 mRNA (Fig. 3A). Afterwards, the ^{32}P-labeled dsRNA products were resolved by SDS-PAGE and quantitated with a PhosphorImager (Fig. 3A). The analysis revealed that all reaction mixtures containing both rVP1 and rVP2 supported the synthesis of dsRNA, while reaction mixtures containing VP1 or VP2 alone did not. However, when the molar ratio of VP1 to VP2 in the reaction mixture was greater

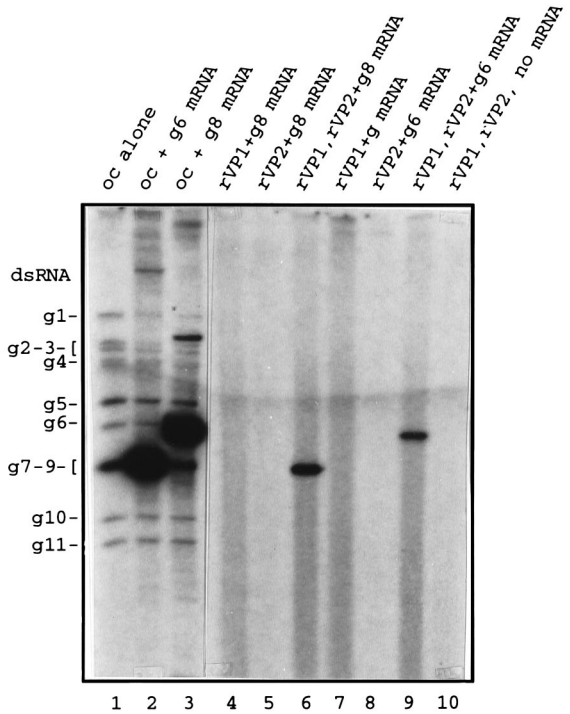

FIG. 2. Synthesis of dsRNA by rVP1 and rVP2. Replicase assays contained SA11-4F open cores (oc) (31), rVP1 (75 μg/ml), and/or rVP2 (50 μg/ml) and either no mRNA or gene 6 or gene 8 mRNA. The reaction mixtures were incubated under the unmodified conditions described in Materials and Methods and were analyzed for ^{32}P-labeled dsRNA by SDS-PAGE and autoradiography. Protein concentrations were determined with a Bio-Rad protein assay kit.

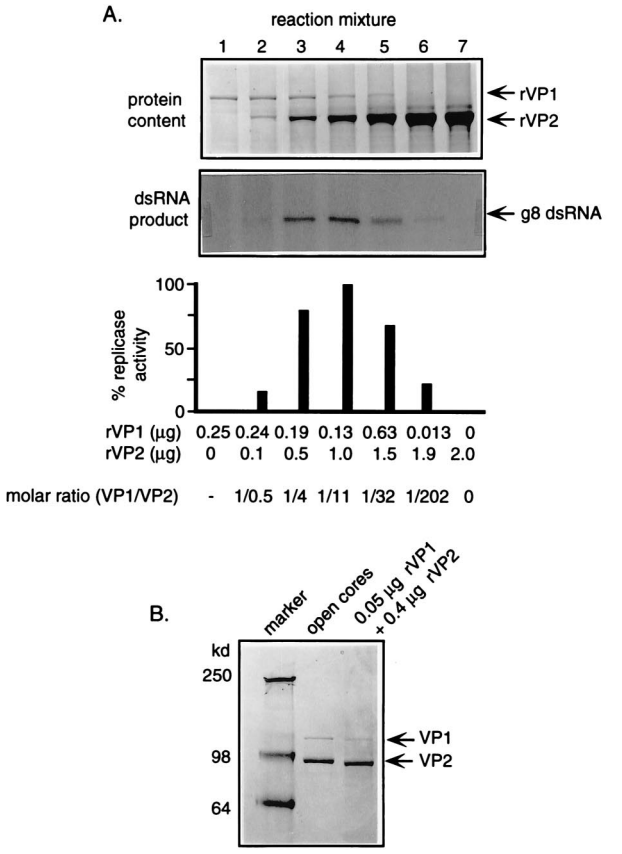

FIG. 3. Ratio of rVP1 and rVP2 producing maximum dsRNA synthesis. (A) Decreasing amounts of rVP1 were incubated with increasing amounts of rVP2 in reaction mixtures (modified conditions) containing gene 8 mRNA. The protein content of each reaction mixture was analyzed by SDS-PAGE and staining with Coomassie blue. ^{32}P-labeled gene 8 dsRNA in the mixtures was detected by SDS-PAGE and autoradiography and quantitated with a PhosphorImager. For ease of comparison, the quantity of ^{32}P-labeled gene 8 dsRNA made in the assay containing 0.13 μg of rVP1 and 1.0 μg of rVP2 was normalized to 100%. The calculated molar ratios were based on a molecular weights of 125,000 for rVP1 and 102,000 for rVP2. (B) Prestained molecular weight markers, open cores, rVP1, and rVP2 were resolved by SDS-PAGE, and the proteins were detected by staining with Coomassie blue. The molar ratio of rVP1 to rVP2 applied to the gel was approximately 1:11.

than 1 (1/0.5) or less than 0.005 (1/202), the level of replicase activity was reduced fivefold or more from maximum levels. The molar ratio of VP1 to VP2 that produced the highest level of dsRNA synthesis in the cell-free system was 1/11, which approximates the molar ratio of VP1 to VP2 predicted to exist in virion cores (1/10) (Fig. 3A) (9, 23, 35, 40). Indeed, electrophoretic analysis confirmed that the molar ratio of rVP1 to rVP2 that catalyzes maximum level of RNA replication was similar to that found in virion cores (Fig. 3B).

Differences between the replicase activities associated with rVP1 and rVP2 and open cores. The effects of mono- and divalent cations and temperature on the replicase activities of rVP1 and rVP2 were analyzed by quantifying the amount of ^{32}P-labeled dsRNA produced in reaction mixtures containing from 0 to 30 mM $MgCl_2$ and 0 to 150 mM NaCl and when incubated at 23 to 45°C. Consistent with assays performed with open cores (5), the replicase of rVP1 and rVP2 was inactive in the absence of magnesium ions (Fig. 4A). But unlike open core assays, where synthesis of dsRNA was maximal at 5 mM magnesium (5), the synthesis of dsRNA by rVP1 and rVP2 was maximal at remarkably high concentrations of magnesium (25 to 30 mM). Indeed, the level of replicase activity associated with rVP1 and rVP2 was at least 10-fold higher at 25 to 30 mM magnesium than at 5 mM magnesium (Fig. 4A). Similar to what was found for the replicase associated with open cores, the replicase of rVP1 and rVP2 was active in the absence of added NaCl (Fig. 4B). When the level of NaCl was increased to up to 100 mM in reaction mixtures, the replicase activity of rVP1 and rVP2 was not inhibited, and at 150 mM NaCl, the activity was decreased by approximately 50%. This is in contrast to assays performed with open cores, where the synthesis of dsRNA is inhibited 10-fold in the presence of 100 mM NaCl and is completely inactive when 150 mM NaCl is included in assays (4a, 5).

While the rotavirus replicase and transcriptase are thought to reflect two different forms of polymerase activity associated with VP1, comparison of the temperature optima of these activities reveals significant differences. In replicase assays performed with rVP1 and rVP2, the synthesis of dsRNA was maximal at 30 to 37°C but was reduced to near background levels at 45°C (Fig. 4C). In contrast, the synthesis of mRNA by the viral transcriptase is not suppressed by high temperatures but indeed is stimulated by incubation at a temperature of 45°C (7).

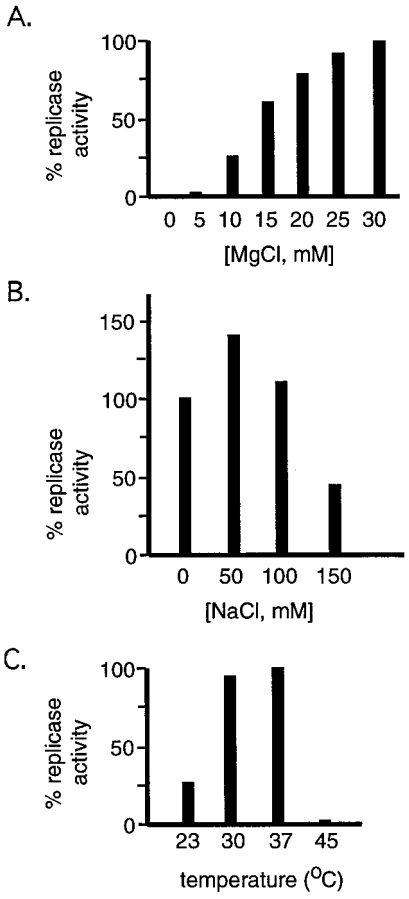

FIG. 4. Optimal parameters for dsRNA synthesis. Replicase assays were performed with rVP1 and rVP2 and gene 8 mRNA under the unmodified conditions described in Materials and Methods except that reaction mixtures contained the indicated final concentration of $MgCl_2$ (A) or NaCl (B) or were incubated at the indicated temperature (C). The products of the assays were resolved by SDS-PAGE, and the amount of ^{32}P-labeled gene 8 dsRNA was quantitated with a PhosphorImager.

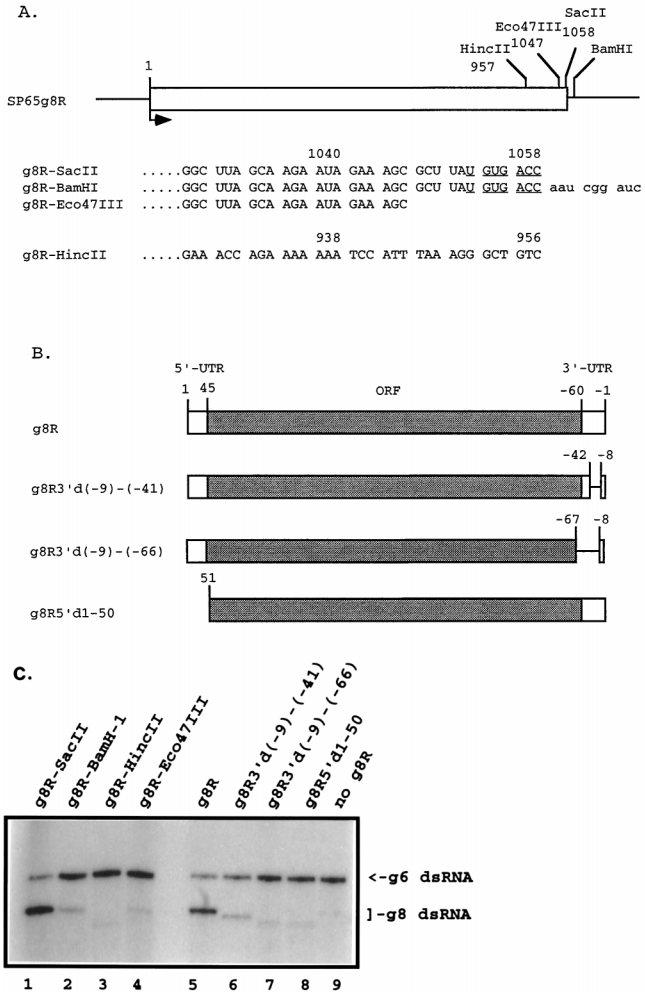

FIG. 5. Role of the termini of viral mRNA in replication by rVP1 and rVP2. (A) T7 transcription vectors were linearized with restrictions enzymes and used in transcription reactions to produce wild-type gene 8 mRNA (g8R-SacII), a mutant gene 8 mRNA containing a 3′-terminal extension of nonviral origin, and two mutant gene 8 mRNAs containing 3′-terminal truncations (g8R-HincII and g8R-Eco47III). The 3′-terminal sequences of the wild-type and mutant gene 8 mRNAs are shown; the 3′ essential *cis*-acting replication signal is underlined, and non-virus-specific sequences are shown in lowercase letters. (B) DNA templates used for the synthesis of gene 8 mRNAs containing deletions within the 3′ UTR [g8R3′d(-9)-(-41) and g8R3′d(-9)-(-66)] and a deletion of the 5′ UTR (g8R5′d1-50) were made by PCR. ORF, open reading frame. (C) Transcripts synthesized from linearized SP65g8R (lanes 1 to 4) and from cDNA templates made by PCR (lanes 5 to 8) were included in replicase assays (modified conditions) at a final concentration of 100 μg/ml. The replicase assays also included 100 μg of wild-type gene 6 mRNA per ml as an internal control, rVP1, and rVP2. The ^{32}P-labeled dsRNA products were detected by SDS-PAGE and autoradiography.

Specificity of replicase activity. The last seven nucleotides of the viral message form a *cis*-acting signal that is essential for minus-strand synthesis by the replicase of open cores (31, 47). To analyze the importance of this signal in the synthesis of dsRNA by rVP1 and rVP2, wild-type gene 8 mRNA (g8R-SacII) and gene 8 mRNA containing a 3′ extension of 9 nucleotides of nonviral origin (g8R-BamH-1) or a deletion of the last 12 (g8R-Eco47III) or 100 nucleotides (g8R-HincII) of the wild-type message were prepared by T7 transcription (Fig. 5A). The wild-type and mutant RNAs were then assayed for the ability to function as templates for dsRNA synthesis in reaction mixtures containing the recombinant proteins and, as competitor, wild-type gene 6 mRNA. As was found previously in assays with open cores (31, 47), both the addition of nucleotides to the 3′ end and deletion of nucleotides from the 3′ end interfered with the ability of the gene 8 mRNA to serve as a template for minus-strand synthesis (Fig. 5C, lanes 1 to 4).

To define the minimum nucleotide sequence at the 3′ end of the gene 8 mRNA that is essential for replication by rVP1 and rVP2, the transcription vector pCITE was used to synthesize a set of RNAs of which the first 549 nucleotides were of nonrotavirus origin and the last 4, 6, 7, 10, 20, or 40 nucleotides were identical to the sequence present at the 3′ end of the gene 8 mRNA (Fig. 6A). The pCITE-gene 8 chimeric transcripts were then assayed for the ability to support minus-strand synthesis in reaction mixtures containing rVP1 and rVP2. In agreement with the results reported with open cores (31), those chimeric RNAs ending with at least the last seven nucleotides found at the 3′ end of the gene 8 mRNA supported efficient replication (Fig. 6B, lanes 1 to 4). In contrast, chimeric RNAs ending with a gene 8-specific sequence of six or fewer nucleotides replicated to only trace levels (Fig. 6B, lanes 5 and 6).

Two *cis*-acting signals that, although not essential for minus-strand synthesis, do function to enhance this process are present in the rotavirus gene 8 mRNA. One of these enhancement signals is located in the 5′ UTR, while the other is located near the 5′ end of the 3′ UTR. To determine whether these enhancement signals functioned to promote RNA replication in assays containing rVP1 and rVP2, we prepared mutant gene 8 mRNAs that lacked the entire 5′ UTR (g8R5′d1-50), the entire 3′ UTR except for the last eight nucleotides of the wild-type message (which contains the 3′ essential replication signal) [g8R3′d(-9)-(-67)], or an internal portion of the 3′ UTR [g8R3′-d(-9)-(-41)] (Fig. 5B). Wild-type and mutant gene 8 mRNAs were then incubated along with gene 6 competitor mRNA in reaction mixtures containing rVP1 and rVP2. Analysis of the dsRNA products of these reactions revealed that

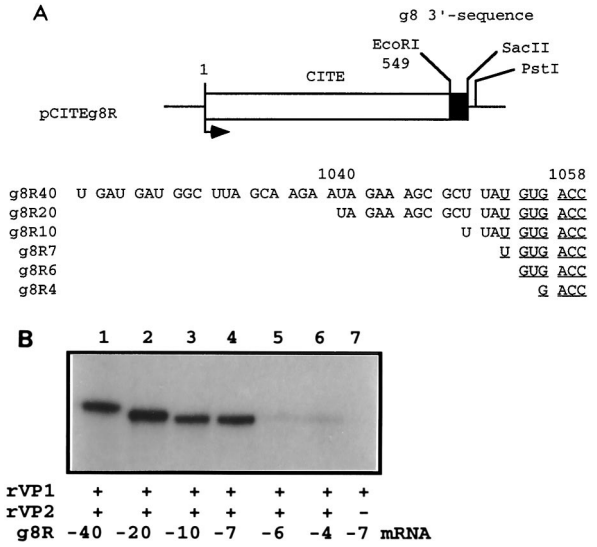

FIG. 6. Recognition of the 3′ essential *cis*-acting replication sequence by rVP1 and rVP2. (A) Sequences representing the last 4 to 40 nucleotides of the gene 8 mRNA were introduced between the *Eco*RI and *Sac*II sites of the transcription vector pCITE. The pCITEg8R constructs were linearized with *Sac*II and transcribed with T7 RNA polymerase to produce the g8R-40, -20, -10, -7, -6, and -4 RNAs. The 3′ essential *cis*-acting replication signal is underlined. (B) The indicated pCITE g8R RNAs were included with rVP1 and rVP2 (lanes 1 to 6) or rVP1 alone (lane 7) in replicase assays (modified conditions). The ^{32}P-labeled dsRNA products were detected by SDS-PAGE and autoradiography.

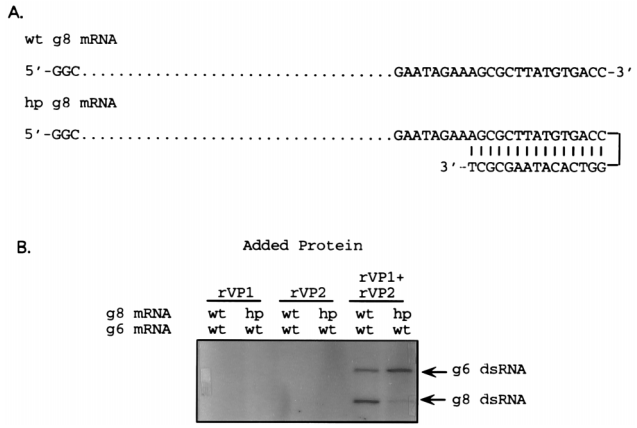

FIG. 7. Assay for primer extension activity of rVP1 and rVP2. (A) Wild-type (wt) gene 8 mRNA and a mutant gene 8 mRNA containing a self-complementary 3′ extension of 15 nucleotides (hp) were produced by T7 transcription of template cDNAs generated by PCR. The predicted hairpin structure formed by the 3′ extension of the mutant mRNA is shown. (B) The wt and hp gene 8 mRNAs were included in replicase assays (modified conditions) containing wild-type gene 6 mRNA, as an internal control, and rVP1 and/or rVP2. The dsRNA products of the assays were detected by SDS-PAGE and autoradiography.

compared to wild-type gene 8 mRNA, all three mutant mRNAs were defective in the ability to serve as templates for minus-strand RNA synthesis (Fig. 5C, lanes 5 to 9). In the case of the mutant RNAs lacking the 5′ UTR (g8R5′d1-50) and lacking all but the last eight nucleotides of the 3′ UTR [g8R3′d(-9)-(-67)], synthesis of only trace levels of dsRNA was detected. In sum, the results establish that the replicase in reaction mixtures containing rVP1 and rVP2 recognizes the same three *cis*-acting replication signals in gene 8 mRNA that were identified before with open cores (31, 47). The data indicate that VP3 plays no role in the recognition of the *cis*-acting replication signals that promote minus-strand synthesis.

Role of VP1 and VP2 in elongation. The synthesis of dsRNA in vitro requires the viral replicase to support both the initiation and the elongation of minus-strand RNA on the mRNA template. As a test of whether VP1 could support elongation in the absence of VP2, we prepared a gene 8 transcript that contained a 3′ extension of 15 nucleotides which was inversely complementary to the 3′-terminal sequence of the wild-type gene 8 mRNA (gene 8HP mRNA) (Fig. 7A). The complementary nature of the two sequences is predicted to lead to the formation of a hairpin structure at the 3′ end of the mRNA which could serve as a primer for minus-strand synthesis and bypass the requirement for initiation in the synthesis of gene 8 dsRNA. The ability of gene 8HP mRNA to catalyze the synthesis of dsRNA was tested by incubation in reaction mixtures that contained either rVP1 or rVP2 or both rVP1 and rVP2. The results showed that despite the presence of the 3′-hairpin primer, neither rVP1 nor rVP2 alone was able to direct the synthesis of dsRNA from the gene 8HP mRNA, and thus neither protein has the primer-dependent activity that is characteristic of RNA polymerases of some other RNA viruses (10) (Fig. 7B).

Interestingly, while dsRNA was produced in reaction mixtures containing rVP1, rVP2, and gene 8HP mRNA, the level of dsRNA synthesis was four- to fivefold less than observed in reaction mixtures containing rVP1, rVP2, and wild-type gene 8 mRNA (Fig. 7B). Hence, while the synthesis of dsRNA from gene 8HP mRNA required rVP1 and rVP2, the fact that the level of dsRNA synthesis was reduced indicates that the presence of the 3′ hairpin interfered with minus-strand synthesis. This finding suggests that efficient elongation of the minus-sense strand on the mRNA template is linked to bona fide initiation occurring on the same template.

Binding of RNA to VP2. Results indicating that VP2 is essential for RNA replication led us to explore the role of the single- and double-stranded RNA-binding activity of the protein on the synthesis of dsRNA (20). To test whether rVP2 bound the mRNA template for minus-strand synthesis, ^{32}P-labeled gene 8 mRNA was incubated with or without rVP2 under the reaction conditions used to assay for replicase activity. rVP1 was not included in the reaction mixtures to prevent minus-strand synthesis from occurring in the presence of rVP2. The components of the reaction mixtures were then resolved by centrifugation on 15 to 30% sucrose gradients. Fractions from the gradients were analyzed for the presence of ^{32}P-labeled mRNA by electrophoresis on nondenaturing polyacrylamide gels under conditions in which RNA-protein complexes are maintained. The results showed that for the reaction mixture lacking rVP2, nearly all of the ^{32}P-labeled gene 8 mRNA remained at or near the top of the gradient and was able to migrate into the nondenaturing gel (Fig. 8A). In contrast, for the reaction mixture containing rVP2, the ^{32}P-labeled RNA was found exclusively in the pellet of the gradient (Fig. 8B) and was unable to migrate into the nondenaturing gel unless the pellet fraction was first deproteinized by phenol-chloroform extraction (Fig. 8C). Analysis of the gradient fractions for protein content by SDS-PAGE revealed that rVP2 was present only in the pellet (not shown). Together, these results indicate that in the cell-free replication system, the mRNA template stably associates with rVP2.

To determine whether the dsRNA product of replicase assays was bound to protein, a reaction mixture programmed to synthesize ^{32}P-labeled dsRNA by the addition of rVP1, rVP2, and gene 8 mRNA was centrifuged on a 15 to 30% sucrose gradient. Fractions from the gradient were then analyzed for ^{32}P-labeled dsRNA and rVP1 and rVP2 by SDS-PAGE. The results showed that nearly 80% of the dsRNA product remained at or near the top of the gradient, while the remaining dsRNA product was present in the pellet (Fig. 9). All of the detectable rVP1 and rVP2 was found in the pellet of the gradient (not shown). Electrophoretic analysis showed that the ^{32}P-labeled dsRNA in the upper gradient fractions was able to migrate into nondenaturing gels, hence indicating that the dsRNA was not bound to protein (Fig. 9). Given that neither rVP1 nor rVP2 was detected in the upper region of the gradient but most of the dsRNA product was, these results suggest that while rVP2 has strong affinity for the mRNA template for replication, the protein lacks appreciable affinity for the dsRNA product of replication under conditions in which the viral replicase is active. These results also indicate that little if any of the dsRNA product of the cell-free replication system undergoes packaging. Indeed, even though some of the dsRNA product (<20%) comigrated with rVP1 and rVP2 to the pellet of the sucrose gradient, the dsRNA seemed only loosely associated with protein, as upon electrophoresis under nondenaturing conditions, it comigrated into the gel with the dsRNA product found at the top of the gradient (Fig. 9).

Importance of mRNA binding to the function of VP2 in replication. Labbe et al. (20) have mapped the RNA-binding domain to the first 132 amino acids of VP2 and have shown by Northwestern blot assay that the protein no longer binds RNA if the first 26 amino acids of the protein are deleted. To address the importance of the RNA-binding activity of VP2 to the protein's essential role in minus-strand synthesis, we generated an rBV which produced VP2 containing an NH_2-terminal de-

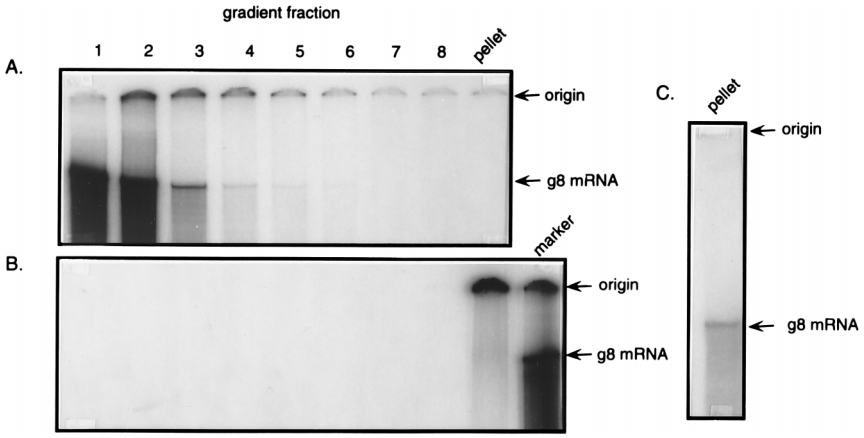

FIG. 8. Affinity of rVP2 for the mRNA template in replicase assays. (A) ^{32}P-labeled gene 8 mRNA was centrifuged on a 15 to 30% sucrose gradient and fractions from the gradient and the pellet analyzed for the presence of radiolabeled RNA by electrophoresis on a nondenaturing 8% polyacrylamide gel and autoradiography. (B) Similar analysis of a ^{32}P-labeled gene 8 mRNA incubated with rVP2 in a replicase assay (modified conditions) prior to centrifugation. (C) The pellet material of the gradient described for panel B was deproteinized by phenol-chloroform extraction and then analyzed by electrophoresis on a nondenaturing gel and autoradiography.

letion of 26 amino acids (rVP2dN26) of the wild-type protein. rVP2dN26 was expressed and purified in the same manner as wild-type rVP2 and, due to the NH_2-terminal deletion, migrated slightly ahead of the wild-type protein upon SDS-PAGE (Fig. 10A). Sucrose gradient centrifugation of coincubated rVP2dN26 and ^{32}P-labeled gene 8 mRNA confirmed that deletion of 26 amino acids from the NH_2 end of VP2 reduces (but does not eliminate) the RNA-binding activity of the protein (data not shown). The ability of a reaction mixture containing rVP1 and rVP2dN26 to support dsRNA synthesis was compared to that of a reaction mixture containing rVP1 and rVP2. As shown in Fig. 10B, the synthesis of dsRNA was inefficient in the presence of rVP2dN26, establishing a correlation between the mRNA-binding activity of VP2 and the capacity of the protein to support minus-strand synthesis in vitro.

DISCUSSION

By analysis of baculovirus-expressed recombinant protein, we have demonstrated that both VP1 and VP2 are necessary and sufficient for the synthesis of rotavirus dsRNA in vitro. Hence, although VP1 contains sequence motifs common to RNA polymerases (4, 43), binds nucleotides (44), and is an essential component of CLPs with replicase activity (49), VP1 lacks polymerase activity in the absence of VP2. Our results showed that even when presented with a viral template mRNA containing a 3′-hairpin primer for the synthesis of minus-strand RNA, VP1 still lacks polymerase activity. This finding suggests that VP1 is unlike the RNA polymerase of poliovirus, a prototypic positive-strand RNA virus which, in the presence of a primer-linked template RNA, can carry out elongation of the minus-strand RNA (10). The observation that VP2, a protein able to self-assemble into CLPs, is essential for minus-strand synthesis supports the hypothesis that RNA replication and encapsidation occur concurrently in rotavirus-infected cells or that RNA replication follows the encapsidation of viral mRNAs. The fact that free pools of dsRNA have not been detected in infected cells further supports the hypothesis that replication and encapsidation of rotavirus RNA are linked events. The mechanism of rotavirus genome replication shows interesting parallels to that of the hepadnaviruses. The viral polymerase, P, of the hepadnaviruses interacts specifically with a *cis*-acting signal of the pregenomic RNA to form a complex which in turn is encapsidated by the core protein C (13). It is within this icosahedral particle that P is then able to catalyze the synthesis of DNA through its reverse transcriptase activity (13, 41). Hence both VP1 and P are able to specifically bind to the template RNA in the absence of other proteins, but despite this interaction they both lack polymerase activity in the absence of the core protein that is responsible for encapsidating the product of replication. Like VP2, C has the ability to self-assemble into particles and binds RNA nonspecifically (13).

While the results demonstrated that VP2 is essential for replicase activity, they also showed that little if any of the dsRNA product made by rVP1 and rVP2 in vitro was packaged. From this, we conclude that the requirements for packaging differ from and are presumably more complex than the requirements for genome replication. The inability of the dsRNA product made by rVP1 and rVP2 to be packaged is due not just to the absence of VP3, given that replicase assays performed with open cores similarly lack the capacity to package their dsRNA product (not shown). Besides VP1 and VP2, other viral proteins including VP6, NSP2, and NSP5 have been shown to accumulate in cytoplasmic inclusions, i.e., viroplasms,

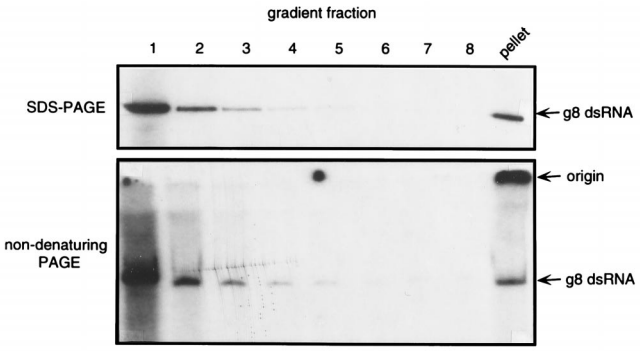

FIG. 9. Assay for binding of the dsRNA product to protein. Gene 8 mRNA, rVP1, and rVP2 were incubated in a replicase assay (modified conditions), and subsequently the reaction mixture was resolved by centrifugation on a 15 to 30% sucrose gradient. Fractions collected from the gradient and the gradient pellet were analyzed for presence of ^{32}P-labeled dsRNA by denaturing (SDS) and nondenaturing PAGE and autoradiography.

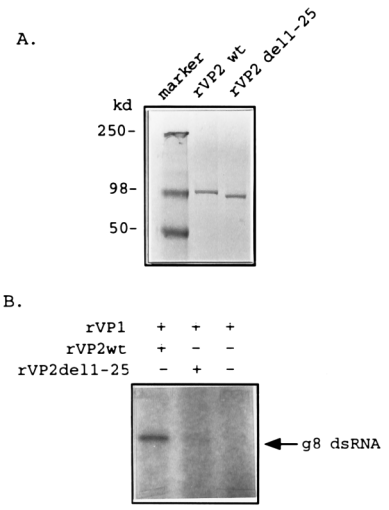

FIG. 10. Importance of the RNA-binding domain of VP2 in dsRNA synthesis. (A) Preparations of wild-type (wt) rVP2 and mutant rVP2 containing an amino-terminal truncation of 25 amino acids (del1-25) were analyzed by SDS-PAGE. (B) Replicase assays (modified conditions) contained wild-type gene 8 mRNA, rVP1, and wt or del1-25 rVP2 and were analyzed for ^{32}P-labeled dsRNA by SDS-PAGE and autoradiography.

that form in infected cells (1, 32). Because viroplasms are thought to be the sites of genome replication and core assembly, perhaps one or more of these other viroplasmic proteins may also participate in encapsidation. NSP2 in particular seems likely to be involved, as earlier studies have shown that empty virions accumulate and that the formation of viroplasms and synthesis of dsRNA is inhibited in cells infected with *ts*E, a mutant virus with a *ts* lesion mapping to the NSP2 gene (14, 36). Such findings and the fact that NSP2 interacts with VP1 and RNA (17), and is a component of replication intermediates with replicase activity (12, 30), suggest that NSP2 may promote RNA replication and packaging.

In addition to other viral proteins, packaging of viral RNA may also require the presence of all 11 viral mRNAs. The pentamers that make up the core have been proposed to function as polymerase units with each pentamer consisting of one species of viral RNA, one copy each of the polymerase VP1 and guanylyltransferase VP3, and five dimers of VP2 (22). Through the interaction of pentamers that contain different viral mRNAs and that exhibit replicase activity, a core which contains the full complement of dsRNAs may be assembled. Indeed, analysis of replication intermediates purified from rotavirus-infected cells have shown that core-like structures are present which have associated replicase activity (12, 30). In replicase assays performed with recombinant proteins or with open cores where only one type of viral mRNA is present, it is possible that since only a single type of pentamer can be formed, the dsRNA product made by the replicase is not packaged because the other types of pentamers are not available to assemble cores. The molar ratio of VP1 to rVP2 found to stimulate the highest level of RNA replication in vitro approximated that of VP1 to VP2 (1:10) proposed for the pentamer of the core. This finding is consistent with the idea that replicase activity is associated with pentamers formed from rVP1 and rVP2 in the cell-free system. Although electron microscopy showed that the rVP2 can self-assemble into CLPs, such particles were not detectable in all preparations of rVP2 even though all such preparations stimulated replicase activity when combined with rVP1. This observation implies that in replicase assays containing rVP1 and rVP2, the synthesis of dsRNA may not be associated with CLPs but instead may be associated with pentameric complexes formed by the recombinant proteins. However, we cannot rule out the possibility that CLPs were present in all preparations of rVP2 but in some cases at concentrations too low to be detected by electron microscopy.

Our results show that viral mRNA as opposed to dsRNA efficiently binds to VP2 and that VP2 containing a deletion in its NH_2-terminal RNA-binding domain no longer cooperates with VP1 to generate replicase activity. These data indicate that binding of the mRNA template to the RNA-binding domain of VP2 is essential for RNA replication. However, based on studies of *ts*F, the presence of a functional RNA-binding domain is not the only aspect of VP2 that is essential for RNA replication. VP2 encoded by *ts*F contains a wild-type RNA-binding domain and has a *ts* A→D mutation at residue 387 (26). Given that core formation is defective in *ts*F-infected cells at the restrictive temperature, these data suggest that RNA replication requires VP2 not only to bind the mRNA template but also to undergo multimerization (25, 36). It is not clear what aspect of core formation is defective for *ts*F VP2, but it may be dimerization, assembly of pentamers from dimers, or assembly of cores from pentamers. Our results are consistent with the idea that for RNA replication to occur, VP2 must assemble into pentamers and that such pentamers function as structural platforms on which the mRNA template is passed as it is replicated into dsRNA (22).

Data showing that the dsRNA product does not associate with rVP2 in replicase assays were somewhat unexpected given that previous studies have indicated that VP2 binds dsRNA by Northwestern blot assay (20) and that the dsRNA genome is intimately associated with the VP2 shell of the core (35). In contrast, our data suggest that under conditions where the viral replicase is active and therefore VP2 is functional, VP2 lacks significant dsRNA-binding activity. The fact that exposure to hypotonic solutions causes cores to become open and to lose their dsRNA genomes also provides evidence that VP2 has at best only weak affinity for dsRNA (5). It is reasonable to conclude from these data that interaction between VP2 and dsRNA is not a major contributing factor that provides stability to the core, nor is it likely that the assembly of the core is driven by the interaction of VP2 with the dsRNA product of replication. The absence of a strong interaction between dsRNA and VP2 is important in considering the viral transcription process, as it would presumably allow the dsRNA template the freedom to rotate and migrate as it is read by the transcriptase.

Despite the absence of VP3, replicase assays performed with rVP1 and rVP2 and with preformed rVP1-rVP2 CLPs were able to synthesize dsRNA (49). Analysis of the *cis*-acting replication signals of viral mRNA indicated that they functioned similarly in reaction mixtures containing rVP1 and rVP2 or open cores. Hence, VP3 neither is an essential component of the replicase nor plays a role in the recognition of the any of the known *cis*-acting replication signals in viral mRNA. Notably, even though VP3 through its guanylyltransferase activity (24, 33) can be expected to interact with the 5′ end of viral mRNA, the absence of VP3 did not affect the activity of the *cis*-acting replication signal located in the 5′ UTR. However, the level of replicase activity associated with rVP1 and rVP2 is significantly less than the level of replicase activity associated with open cores per constant amount of viral protein (data not shown). While the explanation for the difference in replicase activity is not yet known, one possibility is that due to its affinity for VP1, VP2, and/or mRNA, VP3 increases the rate at which these complexes are assembled into structures that have rep-

licase activity in vitro. By analysis of cells infected with *ts*B, a mutant with a *ts* lesion in VP3, Vasquez et al. (45) have also obtained evidence that VP3 plays a role in the formation of replication intermediates with replicase activity, a function which appears to be distinct from the capping function of the protein.

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