

## Template-Dependent, In Vitro Replication of Rotavirus RNA

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**A template-dependent, in vitro rotavirus RNA replication system was established. The system initiated and synthesized full-length double-stranded RNAs on rotavirus positive-sense template RNAs. Native rotavirus mRNAs or in vitro transcripts, with bona fide 3' and 5' termini, derived from rotavirus cDNAs functioned as templates. Replicase activity was associated with a subviral particle containing VP1, VP2, and VP3 and was derived from native virions or baculovirus coexpression of rotavirus genes. A cis-acting signal involved in replication was localized within the 26 3'-terminal nucleotides of a reporter template RNA. Various biochemical and biophysical parameters affecting the efficiency of replication were examined to optimize the replication system. A replication system capable of in vitro initiation has not been previously described for *Reoviridae*.**

Members of the virus family *Reoviridae* have as one of their unique characteristics a genome that consists of multiple segments of double-stranded RNA (dsRNA). The life cycle of the various members of the *Reoviridae* is also unique and can be summarized as follows (10, 16, 35). The virus enters the host cell and replicates in the cytoplasm. After removal of the outermost layer of capsid proteins, the subviral particle-associated transcriptase is activated and produces single-stranded RNAs (ssRNAs) of messenger or positive sense which are extruded from the particle into the cytoplasm. The ssRNAs can function as the message for the synthesis of protein or as a template upon which progeny dsRNA genomes are made by synthesis of the complementary negative-sense strand. The synthesis of the negative-sense strand on the positive-sense template is called replication, and the enzyme catalyzing negative-sense synthesis is called the replicase. Replication occurs in particles that are assembled from newly synthesized viral protein and positive-sense template RNAs. After synthesis of the progeny dsRNA, additional viral proteins are added as the progeny particle matures. Perhaps the most unusual feature of this replication pathway is that both transcription and replication occur in particles. Active transcriptase or replicase have not been solubilized from the particles.

Rotaviruses follow the general replication pathway described above. However, little is known of the molecular details of rotavirus RNA replication since only a single unconfirmed report of template-dependent, in vitro replication has been made (29). Our current understanding of rotavirus RNA replication comes from analysis of subviral complexes (replicase particles) that were isolated from infected cells and were capable of completing nascent negative-strand synthesis in vitro (20, 29). Characterization of the replicase particles (15, 30, 31) showed that a series of particles (replication intermediates [RIs]) that appear to represent a morphogenic pathway

exist and suggested that morphogenesis and replication are intimately related processes. All the RIs contained a full complement of positive-sense template RNAs and were capable of replicase activity. The precore RI, which was the smallest and earliest formed in pulse-labels, contained the structural proteins VP1 and VP3 together with nonstructural proteins NSP1 (NS53), NSP2 (NS35), NSP3 (NS34), and NSP5 (NS26). The precore RI could be chased into the core RI to which VP2 had been added and from which NSP1 was lost. The core RI could subsequently be chased into single-shell (SS) RIs that contained VP1, VP2, VP3, and VP6 and much reduced amounts of NSP2, NSP3, and NSP5. The positive-sense RNA template appeared to extend from the surface of the earliest particles and be drawn into the particle as replication proceeded (31). The precore RI was the earliest particle identified that would support replication of rotavirus RNA. This is plausible given that VP1 contains motifs associated with RNA polymerases (7) and VP3 contains GTP-binding motifs and activity (22, 23). It is also plausible that later particles containing VP2 also had replicase activity since VP2 temperature-sensitive mutants reduce replicase particle activity 20-fold at a nonpermissive temperature (24) and are negative for dsRNA synthesis (3).

From the above discussion, it is clear that in rotavirus, replication (i) occurs in a particulate replicase and (ii) utilizes positive-sense RNA as a template for replication of the negative-sense strand. However, the precise roles of the structural and nonstructural protein components of the replicase particle are not known, and little has been learned about the cis-acting signals on the template RNAs that govern their replication beyond a requirement for the 19 3'-terminal nucleotides (nt) (17). In addition, nothing is known about the cis-acting signals on the template RNAs that regulate binding to the replicase and packaging of the template or its replicated product. Here, we describe a template-dependent, in vitro rotavirus replication system that will allow this information to be obtained.

### MATERIALS AND METHODS

**Cells and viruses.** MA 104 monkey kidney cells were grown in medium 199 containing 5% fetal bovine serum and used for

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rotavirus propagation as described elsewhere (33). *Spodoptera frugiperda* (Sf9) insect cells were grown in Hink's medium, containing 10% fetal bovine serum and used for propagation of recombinant baculoviruses as previously described (11).

Rotavirus strains SA11-4F and B223 were from the laboratory collection and have been described previously (1, 2). Recombinant baculovirus strains expressing the following rotavirus protein species were used: VP1, pVL941/Rf1 (7); VP2, pVL941/Rf2 (21); VP3, pVL1393/SA11-3 (23); and VP6, pAC461/SA11-6 (11).

**Preparation of SA11-4F open-core particles.** Core particles were produced as described previously (6), from SA11-4F purified as described elsewhere (4). The native core particles were opened by dialysis against low-ionic-strength buffer (2 mM Tris [pH 7.6], 0.5 mM EDTA, 0.5 mM dithiothreitol) for 18 h at 4°C. Open cores were sometimes further purified by CsCl gradient centrifugation prior to dialysis into the low-ionic-strength buffer and storage in the presence of 0.01% NaN<sub>3</sub>. Protein concentration was determined with the Bio-Rad protein assay kit.

**Preparation of baculovirus-expressed open-core particles.** Sf9 insect cells were coinfectd at a multiplicity of infection of 5 PFU per cell each with baculovirus recombinants pVL941/Rf1, pVL941/Rf2, pVL1393/SA11-3, and pAC461/SA11-6 as previously described (23, 37). After 2 h of adsorption, the infected cells were pelleted, suspended in Grace's medium containing 0.5% fetal bovine serum, and incubated at 27°C. Six to seven days postinfection, the cells were pelleted from the medium and the supernatant was pelleted through a cushion of 35% sucrose by centrifugation for 90 min at 25,000 rpm in an SW28 rotor. The pellet was suspended and subjected to isopycnic CsCl gradient centrifugation for 18 h at 35,000 rpm in an SW50.1 rotor. The two visible bands were collected by side puncture of the centrifuge tube and dialyzed against Tris-buffered saline. The resulting VP1/2/3/6 particles were converted to open cores as follows. VP6 was removed by dialysis overnight against 1.0 M CaCl<sub>2</sub> at 37°C. The resulting VP1/2/3 particles were opened by dialysis of the core particles into low-ionic-strength buffer (2 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 0.5 mM dithiothreitol) for 18 h. The open VP1/2/3 particles were stored in the presence of 0.01% NaN<sub>3</sub>. The protein concentration was determined as for SA11-4F open cores.

**Preparation of B223 mRNA templates.** B223 SS particles were prepared as previously described (5). Total mRNA was obtained from the B223 SS particles by using the in vitro transcription reaction described by Mason et al. (25) modified by substituting the RNase inhibitor RNasin (200 U/ml) for bentonite. SS particles were removed from the reaction mixture by centrifugation, and the supernatant was adjusted to 1% sodium dodecyl sulfate (SDS) and extracted twice with water-saturated phenol. The aqueous phase was precipitated with ethanol and dissolved and adjusted to 2 M LiCl<sub>2</sub> to precipitate the mRNA away from any dsRNA from broken SS particles. The mRNA precipitate was collected, dissolved, and precipitated with ethanol. The precipitated mRNA was collected and dissolved in H<sub>2</sub>O, and aliquots were stored at -70°C until use. The mRNA concentration was determined spectrophotometrically.

**Construction of the OSU segment 9 transcription vectors.** A transcription vector for the expression of full-length porcine rotavirus strain OSU segment 9 transcripts with precise, native 5' and 3' termini was constructed. A PCR-modified cDNA of OSU segment 9 (GenBank accession number x04613) under the control of the T7 promoter was generated by reverse transcription PCR using the following primers: (i) positive-

sense primer 5'-CCAGGTACCTAATACGACTCACTATA GGCTTTAAAGAGAGAATTTCCGACTGG-3' (the T7 promoter is doubly underlined, the *Kpn*I site for cloning is singly underlined, and the viral sequence is boldfaced) and (ii) negative-sense primer 5'-GGTAAGCTTCCGCGGTCACATC ATACAGTTCTAAC-3' (the *Ksp*I [*Sac*II] site for cleavage to yield a precise 3' end on the transcript is doubly underlined, the *Hind*III site for cloning is singly underlined, and the viral sequence is boldfaced). The PCR-modified OSU segment 9 cDNA was then cloned into the *Kpn*I-*Hind*III site of pUC19 to yield pUC/OSU9. Clones containing the desired insert were confirmed by restriction digestion and sequencing of ~250 nt across each cloning boundary using pUC19 sequencing primers.

An internal deletion construct was derived by a one-step subcloning procedure using the two *Eco*RV sites in the OSU9 insert to remove nt 88 to 599 of the OSU9 sequence. pUC/OSU9 was cut with *Eco*RV, and the plasmid was blunt-end ligated to produce pUC/OSU9-ΔRV.

**Transcription of OSU segment 9 cDNAs.** Plasmids pUC/OSU9 and pUC/OSU9-ΔRV were linearized with *Ksp*I, *Bfi*I, or *Xba*I so that various-length transcripts would be obtained. The 3'-terminal overhang of *Ksp*I-linearized DNA was blunt ended in some cases with T4 DNA polymerase at room temperature for 20 min. The linearized plasmids were then transcribed by using T7 RNA polymerase (Promega) as recommended by the manufacturer. Following transcription reactions, the DNA templates were removed by RQ1 RNase-free DNase (Promega) at 37°C for 15 min, and the transcripts were recovered by ethanol precipitation.

**Initial in vitro replication system.** In the initial experiments, the 30-μl in vitro replication reaction mixture contained 50 mM Tris-HCl (pH 7.8); 10 mM Mg acetate; 1.25 mM (each) ATP, GTP, and CTP; 0.5 mM UTP; 8 mM phosphoenolpyruvate; 50 μg of phosphoenolpyruvate kinase per ml; 1 mM *S*-adenosyl-L-methionine; 2 mM dithiothreitol; 800 U of RNasin per ml; 15 μCi of [ $\alpha$ -<sup>32</sup>P]UTP (3,000 Ci/mM), 3.6 μg of total B223 transcripts, and 0.2 μg of SA11-4F open cores. The reaction was carried out at 37°C for 2 h, and the products were analyzed on SDS-12% polyacrylamide gels.

**Optimization of the in vitro replication system.** The requirement for and optimal concentrations of the various components of the replication system were systematically examined by using open cores from SA11-4F as the source of the replicase and transcripts from pUC/OSU9 as the reporter templates. The kinetics of the reaction were examined by analyzing aliquots taken from a mass reaction at various times of incubation.

**Template specificity of the replicase.** Template specificity was determined by programming the optimized reaction mixture with the following nonrotavirus RNAs: luciferase mRNA, brome mosaic virus RNAs, tobacco ringspot virus RNAs, and T7 transcripts of Norwalk virus RNA. Specificity was examined in the presence of either Mg<sup>2+</sup> or Mn<sup>2+</sup>.

**Denaturing gels of replicase products.** A phenol-extracted <sup>32</sup>P-labeled replicase reaction product or <sup>32</sup>P-labeled marker dsRNA from OSU was dissolved in sample buffer consisting of 80% formamide, 10 mM EDTA, and 1 mg of bromophenol blue per ml in water. Prior to electrophoresis, samples were heated at 37°C to visualize dsRNA and at 100°C to visualize ssRNA. Aliquots of the RNA were subjected to electrophoresis in 5% polyacrylamide gels which contained 7 M urea, 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA. Electrophoresis was carried out for 4.5 h at 450 V.

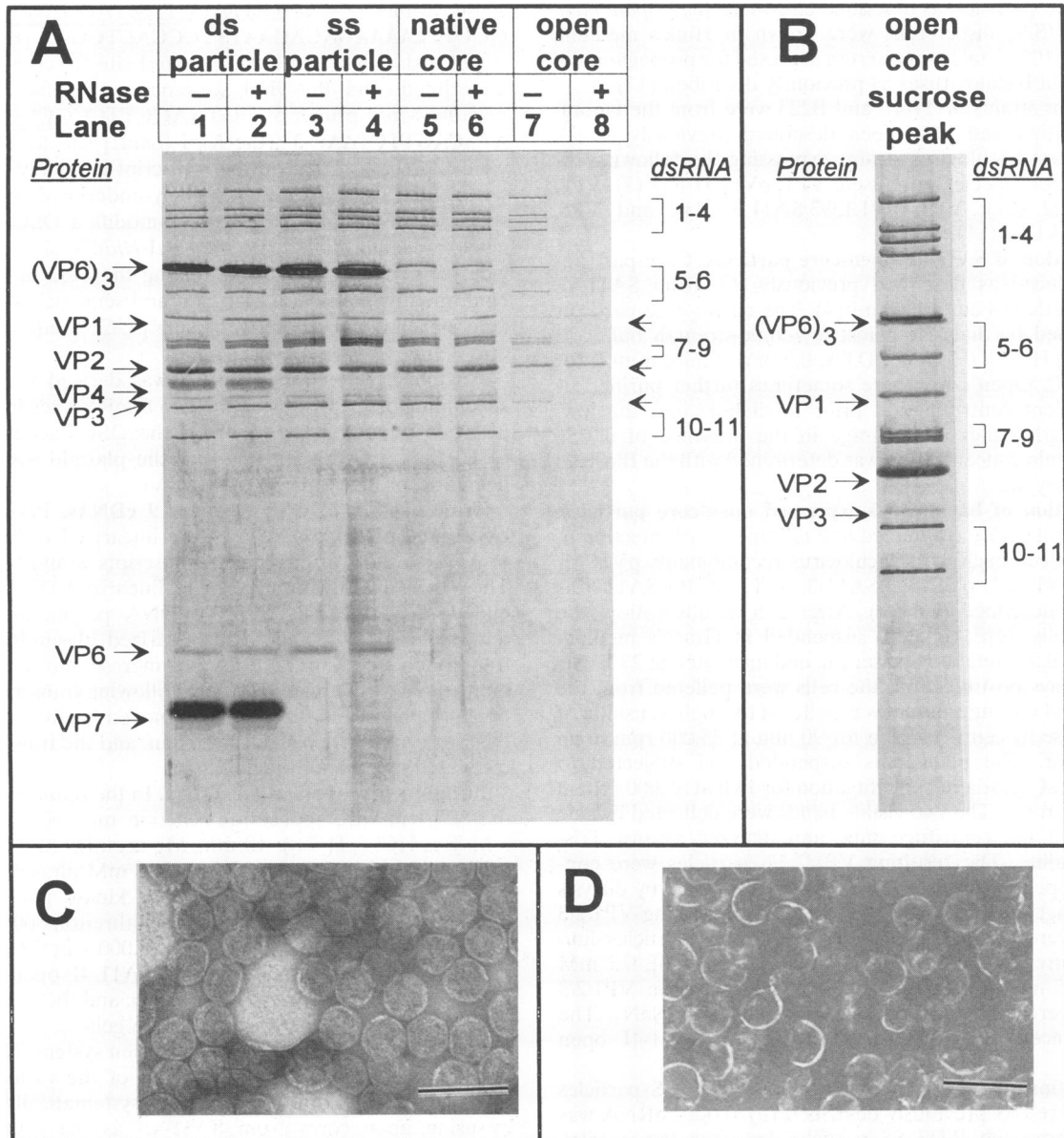


FIG. 1. Preparation and characterization of open cores derived from virions. (A) Silver-stained gel showing the total protein and RNA contents (odd lanes) and RNase susceptibilities of the endogenous genomic RNA (even lanes) of the various purified particles in the stepwise disassembly of SA11-4F virions to prepare open cores. Particles in 150 mM NaCl–25 mM Tris-HCl (pH 7.4) were either treated with RNase A at a final concentration of 40  $\mu$ g/ml for 30 min at 37°C or untreated prior to dissolving in sample buffer and running on the gel. The positions of the rotavirus proteins are indicated on the left, and the positions of the genomic dsRNAs are indicated on the right. Arrows on the right, positions of VP1, VP2, and VP3 in the open cores. The gel was run without boiling the samples, so VP6 runs primarily in its trimeric form [(VP6)<sub>3</sub>]. (B) Silver-stained gel showing the total protein and RNA contents of SA11-4F-derived open cores from the peak fraction of a sucrose gradient. Note the stronger intensities of VP1 and VP3 in this concentrated sample. The trace amount of (VP6)<sub>3</sub> in this preparation was not reproducibly present. (C) Electron micrograph of SA11-4F-derived native cores. Bar, 100 nm. (D) Electron micrograph of SA11-4F-derived open cores. Note the empty appearance of the open-core particles. Bar, 100 nm.

## RESULTS

**Preparation and characterization of rotavirus particles with replicase activity. (i) Open cores from native virions.** Open-core particles were prepared from purified SA11-4F virions by the stepwise disassembly described above. Figure 1A shows the protein and RNA content of each of the particle types generated as well as the RNase A susceptibility of the genomic RNA associated with each particle type. Double-shell particles

contained the normal complement of proteins (lane 1), and the dsRNA was RNase resistant (lane 2). Removal of the outer capsid by EDTA treatment produced normal SS particles in which the dsRNA was RNase resistant (lanes 3 and 4). Treatment with 1 M CaCl<sub>2</sub> removed VP6 and produced core particles in which the dsRNA was RNase resistant (lanes 5 and 6). Dialysis of the core particles against low-ionic-strength buffer for 18 h produced open-core particles in which the

dsRNA was RNase susceptible (lanes 7 and 8). Open-core particles contained protein species VP1, VP2, and VP3 (Fig. 1A [lane 8] and B). It is unclear if the dsRNA of the open cores exits the particle or if the dsRNA is made accessible to RNase by treatment of the cores with low-ionic-strength buffer. However, the association of dsRNA with the open-core particles in sucrose gradients (Fig. 1B) suggests that a portion of the dsRNA remains associated with the particles, while the density of open cores (1.31 to 1.32 g/ml) indicates that a significant portion of the dsRNA has exited since native cores have a density of 1.50 g/ml (6). Electron micrographs of native cores (Fig. 1C) and open cores (Fig. 1D) suggest that the open-core particles are empty. The proteins of open-core particles are subject to digestion with trypsin, whereas those of double-shell, SS, and core particles are not (data not shown). The preparation of open-core particles has replicase activity (see below).

(ii) **Baculovirus-expressed virus-like particles.** The baculovirus-expressed equivalent of SS particles (recombinant-rotavirus [rRV] SS particles) were produced by the coexpression of VP1/2/3/6 in insect cells as described in Materials and Methods. Four rotavirus proteins were coexpressed, as we found that expressed particles containing VP1/2/3 aggregated strongly during purification, as do rRV VP2 particles (37), and required detergent to obtain a monodisperse suspension. In contrast, rRV SS particles (Fig. 2A) could be obtained as a monodisperse suspension (Fig. 2B). Expressed rRV SS particles contained VP1/2/3/6, although much of the VP2 was present in the form of cleavage products (Fig. 2A, arrowheads) that obscured the position of VP3, which was demonstrated to be present by Western blot (immunoblot) and GTP-binding assays (data not shown). The degradation products of VP2 in particles have been described and characterized previously (37). The rRV SS particles formed two narrowly separated but distinct bands in CsCl gradients. The protein contents of both bands were identical to that for the bottom band (Fig. 2A), with the only detectable difference being the relative contents of the cleavage products (data not shown). The rRV SS particles were purified and converted to rRV core particles and then to rRV open cores as for native SS particles. The baculovirus-expressed rRV SS particles appeared to contain no RNA (Fig. 2B), and no endogenous RNA could be detected in gels (data not shown). The resulting rRV open cores have replicase activity (see below).

**Preparation and characterization of RNA templates.** (i) **Native mRNA templates.** mRNA was produced from virus strain B223 by using the endogenous transcriptase of the SS particle. Following extensive purification of the resulting mRNAs, they were tested for integrity by confirming the presence of all 11 full-length mRNAs in gels and by *in vitro* translation, in which they directed the synthesis of all 11 rotavirus protein species (data not shown).

(ii) **Reporter RNA templates.** A cDNA clone of rotavirus strain OSU genome segment 9, under the control of the T7 promoter and having precise 5' and 3' termini, was generated by PCR and cloned into pUC19 as described above. Linearization of the resulting plasmid (pOSU9) with *KspI* (*SacII*) and transcription with T7 polymerase yielded a transcript with precise 5'-GG... and ...CC-3' termini. An internal deletion was constructed by deletion of the *EcoRV* fragment extending from nt 87 to 600 (pOSU9- $\Delta$ RV). Two 3'-terminal truncations were generated by linearizing pOSU9 or pOSU9- $\Delta$ RV with *BfI* (26 nt short) or *XbaI* (37 nt short). The structures of the resulting reporter RNA templates are shown in Fig. 3.

Analysis of the transcripts resulting from these constructs (Fig. 4) showed that linearization of pOSU9 with *KspI* produced transcripts predominantly of the expected 1,062-nt

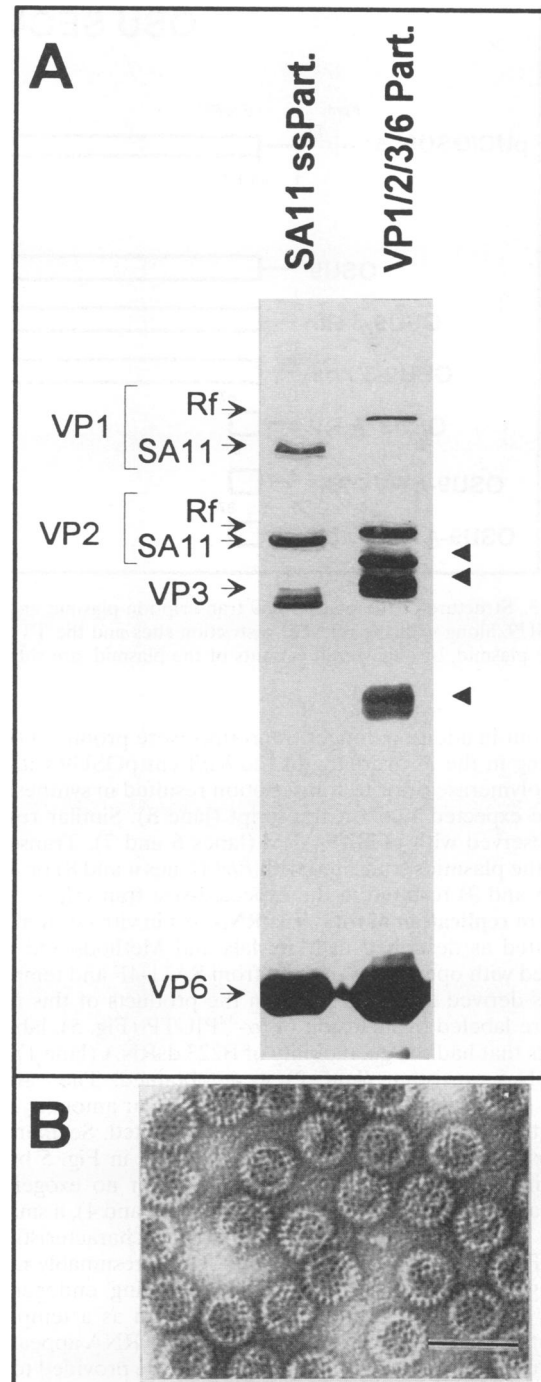


FIG. 2. Characterization of baculovirus-expressed rRV SS particles. (A) Silver-stained gel of rRV SS particles (VP1/2/3/6 Part.) and SA11-4F SS particles. The positions of the viral proteins are indicated on the left. Note that the rRV SS particles contain VP1 and VP2 of lower electrophoretic mobilities because the expressed VP1 and VP2 were from bovine virus strain Rf and have mobilities lower than those of the cognate proteins of SA11-4F. Arrowheads, positions of bands that represent degradation products of VP2 and VP3 (lane VP1/2/3/6 Part.) (37; also, unpublished data). (B) Electron micrograph of purified rRV SS particles. Note the empty appearance of the particles. Bar, 100 nm.

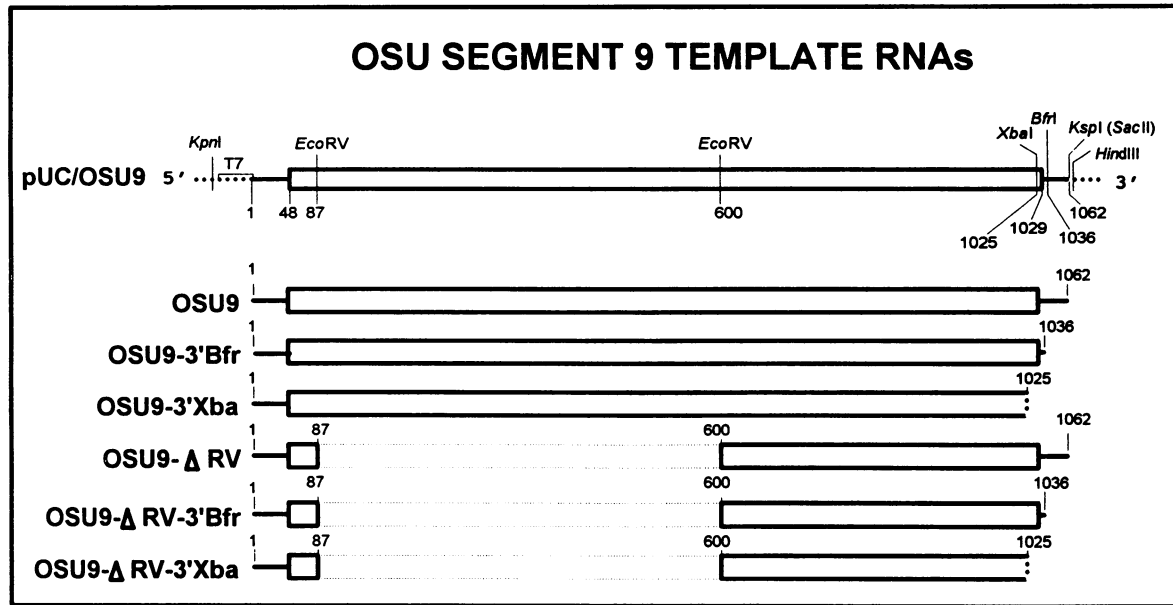


FIG. 3. Structure of the pUC/OSU9 transcription plasmid and the various transcripts derived from variants of that plasmid. The structure of pUC/OSU9, along with the relevant restriction sites and the T7 promoter, is shown at the top. The structures of the various transcripts derived from the plasmid, or engineered variants of the plasmid, are shown below.

length, but in addition, longer transcripts were produced (lane 2). Filling in the 3' overhang of the *KspI*-cut pOSU9 with T4 DNA polymerase prior to transcription resulted in synthesis of only the expected 1,062-nt transcript (lane 3). Similar results were observed with pOSU9- $\Delta$ RV (lanes 6 and 7). Transcription of the plasmids truncated with *BfrI* (lanes 4 and 8) or *XbaI* (lanes 5 and 9) resulted in the expected-size transcripts.

**In vitro replication of rotavirus RNA.** An in vitro system was constituted as described in Materials and Methods and programmed with open cores derived from SA11-4F and template mRNAs derived from B223. When the products of this reaction were labeled by inclusion of [ $\alpha$ - $^{32}$ P]UTP (Fig. 5), labeled products that had the gel mobility of B223 dsRNA (lane 1) and were RNase resistant (lane 2) were obtained. The various dsRNA species were produced in nonequimolar amounts, with segments 2, 5, 6, and 10 being underrepresented. Segment 10 was most underrepresented and is not visible in Fig. 5 but is visible in longer exposures of the gel. When no exogenous RNA was added to the in vitro system (lanes 3 and 4), a smaller amount of dsRNA having the gel mobility characteristic of SA11-4F was synthesized. This dsRNA was presumably made by using the positive strand of the remaining endogenous dsRNA present in the open-core preparation as a template. The synthesis of dsRNA from the endogenous RNA appears to be suppressed if exogenous RNA templates are provided to the system since no dsRNAs with the mobility of the products from the endogenous template were observed when an exogenous template was provided (lanes 1 and 2). When no open cores (lanes 5 and 6) or native cores (lanes 7 and 8) were added to the reaction mixture, no dsRNA products were synthesized. Exogenously added dsRNA did not function as a template (data not shown). Quantitatively similar results were obtained when the label was supplied to the reaction mixture in the form of [ $\alpha$ - $^{32}$ P]CTP or [5,6- $^3$ H]UTP (data not shown). When the label was supplied to a reaction mixture (containing unlabeled nucleoside triphosphates) in the form of [ $\alpha$ - $^{32}$ P]UTP-labeled B223 mRNAs, the reaction products were labeled, demonstrat-

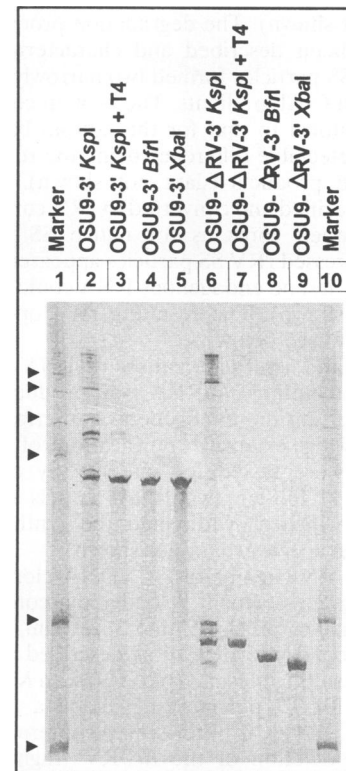


FIG. 4. Transcript RNAs derived from the various pUC/OSU9 constructs. A silver-stained gel of the transcripts derived from the constructs in Fig. 3 is shown. Note the presence of run-on transcripts in templates derived from constructs cut with *KspI* (lanes 2 and 6) and their absence after treatment of the *KspI*-cut plasmid with T4 polymerase to fill in the 3' overhang (lanes 3 and 7). Marker RNAs (lanes 1 and 10) (arrowheads, from the top): 5.3, 2.8, 1.9, 1.2, 0.6, and 0.3 kb.

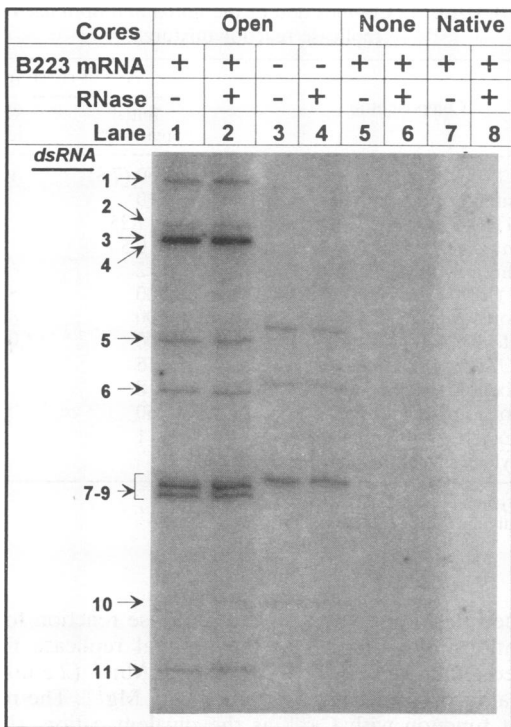


FIG. 5. Products of the in vitro replication reaction programmed with open cores and native viral mRNAs. The in vitro replication system was programmed with the form of the core shown, exogenous B223 was added as indicated, and the <sup>32</sup>P-labeled products were resolved in polyacrylamide gel electrophoresis. Lanes RNase +, RNase-resistant dsRNA products of the reaction programmed with each type of core. The positions of the dsRNAs are indicated on the left. Note the differences between the migration rates and amounts of the dsRNAs replicated on exogenous B223 templates (lanes 1 and 2) and the migration rates and amounts of those replicated on the endogenous templates (lanes 3 and 4).

ing that the mRNA was actually serving as a template in the reaction (data not shown).

**Replication of reporter template RNAs.** To determine if the in vitro replicase would replicate synthetic rotavirus RNA templates, we programmed the system with SA11-4F open cores and various exogenous template RNAs as shown in Fig. 6. When the system was programmed with B223 mRNAs, the standard B223 dsRNAs were made (lane 1). If no exogenous RNA was added to the system, a very weak background level of SA11-4F dsRNA was observed (lane 2). No dsRNA was synthesized in the absence of open cores (lane 3). Programming the system with increasing amounts of the transcript derived from *KspI*-linearized pOSU9 resulted in the synthesis of increasing amounts of dsRNA with the mobility of OSU segment 9 (lanes 4 to 6). Adding increasing amounts of the OSU9 transcript with a constant amount of B223 mRNA resulted in both B223 and OSU segment 9 dsRNAs being synthesized, with the relative amount of OSU segment 9 increasing in parallel with the input of the OSU9 transcript (lanes 7 to 9). When the reaction mixture contained the linearized pOSU9 plasmid from which the OSU9 transcripts were derived, no dsRNA product other than that from the endogenous SA11-4F template was made (lane 10). Since the OSU9 transcript contained precise 5' and 3' termini, these results indicated that synthetic rotavirus RNA templates with precise termini could be replicated.

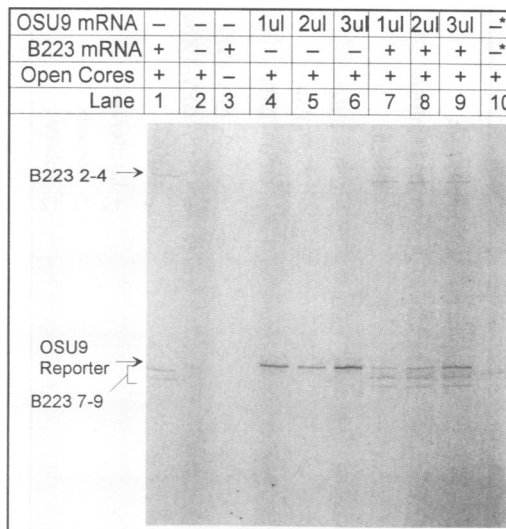


FIG. 6. Replication of reporter templates derived from rotavirus transcription vectors. The in vitro replication system was programmed with virion-derived open cores and native B223 mRNA, synthetic OSU9 template RNA, or both, as indicated. The <sup>32</sup>P-labeled reaction products were treated with RNase prior to resolution in polyacrylamide gel electrophoresis. The positions of the major dsRNA products are indicated on the left. \*, the reaction mixture of lane 10 was programmed with the DNA transcription plasmid from which the OSU9 reporter was derived. Note that, because of the short exposure to film, only the major product bands (2 to 4 and 7 to 9) made in response to the B223 template are visible.

**cis-acting replication signals on template RNA.** Possible locations of replication signals on the template RNAs were examined by programming the in vitro replication system with transcripts of an internal deletion (OSU9-ΔRV) or 3'-terminal truncated transcripts derived from either the full-length OSU9 plasmid or the OSU9-ΔRV plasmid (Fig. 3). The replication system with SA11-4F-derived open cores synthesized B223 dsRNAs when programmed with B223 mRNA (Fig. 7, lane 2) and very slight amounts of SA11-4F dsRNA when no exogenous template was added (lane 1), although this signal is too weak to be seen in Fig. 7. Full-length OSU9 transcripts, derived from *KspI*-linearized pOSU9, programmed the synthesis of OSU genome segment 9 (lane 3). OSU9 transcripts truncated by 26 3' nt by linearization of the transcription plasmid at the *BfrI* site were not detectably replicated (lane 4). Similar results were found for transcripts truncated by 37 3' nt by linearization of the plasmid at the *XbaI* site (lane 5). An internal deletion of OSU9, produced by transcription of pOSU9-ΔRV linearized with *KspI*, was replicated and resulted in a dsRNA 513 bp shorter than that produced from the parental OSU9 transcript (lane 6). The requirement for the 3'-terminal 26 and 33 nt was confirmed by truncation of OSU9-ΔRV transcripts at the *BfrI* and *XbaI* sites, respectively. Neither of the truncated deletion transcripts was replicated (lanes 7 and 8). These results indicate that no *cis*-acting replication signals reside within the *EcoRV* fragment of OSU segment 9 (nt 87 to 600). Furthermore, the failure of transcripts with 3'-terminal truncations of 26 and 33 nt to replicate indicates that *cis*-acting replication signals reside very near the 3' terminus of the OSU9 template.

**Template specificity of the in vitro replication reaction.** The abilities of nonrotavirus RNAs to serve as templates were examined. A number of natural and synthetic ssRNAs were

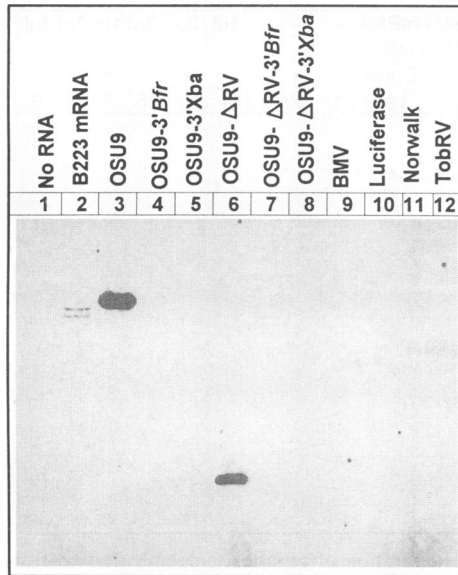


FIG. 7. In vitro replication of synthetic template RNAs containing internal deletions or 3' terminal truncations. The in vitro replication system was programmed with B223 mRNA or transcripts derived from engineered pUC/OSU9 plasmids as indicated. The  $^{32}\text{P}$ -labeled reaction products were treated with RNase prior to resolution in polyacrylamide gel electrophoresis. Note dsRNA replication products of appropriate size for OSU9-3'*KspI* (lane 3) and OSU9- $\Delta$ RV (lane 6) transcripts and the failure of all transcripts with 3'-terminal truncations or foreign RNAs to be replicated. BMV, brome mosaic virus; TobRV, tobacco ringspot virus.

obtained and tested for their abilities to direct the synthesis of the relevant protein, prior to being used to program the in vitro replication system. None of the following RNAs was replicated in the in vitro replication system: brome mosaic virus RNAs, luciferase mRNA, T7 transcripts of Norwalk virus RNA, and tobacco ring spot virus RNAs (Fig. 7, lanes 9 to 12, respectively). The failure of nonrotavirus RNAs to be replicated suggested specific recognition of rotavirus templates by the particulate replicase.

**Optimization of the in vitro replication system.** Our initial in vitro replication system was identical to the in vitro transcription system originally described by Cohen et al. (8), except that exogenous mRNA was added to serve as a template and open cores were added to serve as the source of replicase activity. While this system synthesized dsRNA on mRNA templates (Fig. 5), the incorporation was somewhat low and gel exposures took 2 to 4 days.

We carried out a thorough optimization of the in vitro replication reaction using SA11-4F-derived open cores as the particulate replicase and in vitro-synthesized OSU9 transcripts as the template. The results of this optimization are summarized in Table 1, which compares our initial system with the optimized system. Direct comparison showed that the optimized system incorporated greater than 10-fold more counts into dsRNA than the initial system. With the optimized system, strong bands could be detected in 6- to 12-h exposures.

Several points relative to optimization merit comment (data not shown). (i) Phosphoenolpyruvate, phosphoenolpyruvate kinase, and *S*-adenosyl-L-methionine were not required for activity, and their omission reduced the background of the RNase-resistant material synthesized. (ii) The rotavirus transcriptase has an absolute requirement for  $\text{Mg}^{2+}$  (8). We

TABLE 1. Composition of the initial and optimized replicase reaction mixtures

Component(s)	Amt or concn (pH)	
	Initial reaction <sup>a</sup>	Optimal reaction <sup>b</sup>
Tris-HCl (mM)	50 (7.8)	100 (7.2)
Magnesium acetate (mM)	10	5
ATP, GTP, CTP (mM [each])	1.25	0.1
UTP (mM)	0.5	0.04
Dithiothreitol (mM)	2	2
RNasin (U/ml)	800	800
Open cores (ng)	200	800
Template RNA (ng)	3,600	1,000
[ $\alpha$ - $^{32}\text{P}$ ]UTP ( $\mu\text{Ci}$ )	15	10
Phosphoenolpyruvate (mM)	8	
Phosphoenolpyruvate kinase ( $\mu\text{g/ml}$ )	50	
<i>S</i> -Adenosyl-L-methionine (mM)	1	
Polyethylene glycol 4000 (%)		1.5

<sup>a</sup> Thirty microliters, 120 min, 37°C.

<sup>b</sup> Twenty microliters, 180 min, 35°C.

examined the requirement of the replicase reaction for divalent cations. We found that the optimal replicase reaction required either  $\text{Mg}^{2+}$  (2.5 to 5.0 mM) or  $\text{Mn}^{2+}$  (2.5 mM), but maximal incorporation was obtained with  $\text{Mg}^{2+}$ . The reaction did not function with  $\text{Ca}^{2+}$  as the divalent cation. (iii) The replicase activity was optimal at pH 6.8, and activity was not detectable at pH 8.5. We chose, in our optimal system, to use pH 7.2 so that we could use Tris-HCl buffer with its large buffering capacity. (iv) The replicase activity functioned over a wide temperature range, with the optimum being 33 to 35°C. This is significantly lower than the optimum temperature (45°C) for the SA11-4F transcriptase activity (6a). (v) The replicase activity was optimal at a relatively low salt concentration. Addition of  $\text{Na}^+$  or  $\text{K}^+$ , as either chloride or acetate salts, inhibited the reaction at concentrations as low as 25 mM. (vi) Polyethylene glycol was not absolutely required for replicase activity, but addition of polyethylene glycol 4000 at concentrations up to 1.5% significantly stimulated incorporation. (vii) Kinetic studies showed that in vitro replication products could be detected in as little as 10 min of reaction, and the product continued to accumulate until approximately 4 h of reaction. We chose 3 h as the optimal time for the reaction. (viii) The optimized system utilized capped and uncapped templates with equal efficiency (data not shown).

**Baculovirus-expressed open-core particles have replicase activity.** Baculovirus-expressed rRV open cores were prepared and tested in the optimized in vitro replicase assay for replicase activity. Both top and bottom band particles were tested. As shown in Fig. 8, rRV open cores had no endogenous RNA that was replicated in the absence of exogenous RNA templates (lanes 4 and 7). When exogenous B223 mRNA was added as a template, B223 dsRNAs were synthesized (lanes 5 and 8). If OSU9 transcripts were added as a template, OSU segment 9 dsRNA was synthesized (lanes 6 and 9). This was in contrast to the result obtained with SA11-4F-derived open cores, for which in the absence of exogenous RNA small amounts of SA11-4F dsRNA were synthesized on the endogenous template (lane 1). If B223 mRNA or OSU9 transcripts were supplied, the open cores synthesized a significant amount of B223 dsRNA or OSU segment 9 dsRNA, respectively (lanes 2 and 3). Taken together, these results indicate that a functional in vitro replication system for rotaviruses can be reconstituted from (i) a baculovirus-expressed particulate replicase, (ii) in

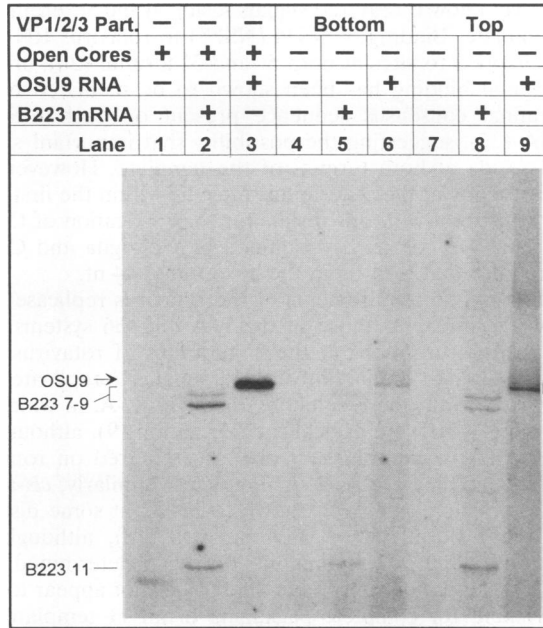


FIG. 8. In vitro replication catalyzed by baculovirus-expressed replicase particles. The optimized in vitro replication system was programmed with either virion-derived open cores or baculovirus-expressed rRV open cores (VP1/2/3). Either an OSU9 template or no template was added. The  $^{32}\text{P}$ -labeled reaction products were run directly in polyacrylamide gel electrophoresis without RNase treatment, although identical results were obtained when the products were treated with RNase. Note the replication of the OSU9 template by the rRV open cores and absence of endogenous dsRNA replication products with the rRV open cores.

vitro-synthesized transcripts of rotavirus cDNAs, and (iii) a simple combination of salts and nucleoside triphosphates. Thus, both the particulate replicase and the template RNAs are subject to genetic manipulation.

**Authenticity of the replication product.** To examine the authenticity of the replication product, the OSU9 template was used to program the in vitro system containing baculovirus-expressed open-core particles and  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ . The reaction product, along with  $^{32}\text{P}$ -labeled OSU genome dsRNA isolated from purified virions, was subjected to electrophoresis on gels containing 7 M urea following sample preparation that results in denaturation ( $100^\circ\text{C}$ ) or no denaturation ( $37^\circ\text{C}$ ) of dsRNAs. As shown in Fig. 9, the nondenatured OSU9 product comigrated with authentic OSU segment 9 (lane 2), while the denatured OSU9 product comigrated with OSU segment 9 ssRNA (lane 4). These results indicate that the negative-strand OSU9 product is a unit length ssRNA and did not result from synthesis of the negative-strand initiating from a snapback hairpin at the 3' terminus of the OSU9 template. We also have preliminary evidence that initiation of the negative strand is de novo, as the product can be labeled with  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  (data not shown).

## DISCUSSION

Prior studies of rotavirus RNA replication in vitro utilized replicase particles isolated from virus-infected cells and resulted in a description of the RNA and protein composition of the various subviral particles capable of synthesizing dsRNA (15, 29–31). *cis*-acting signals on the template RNAs could not

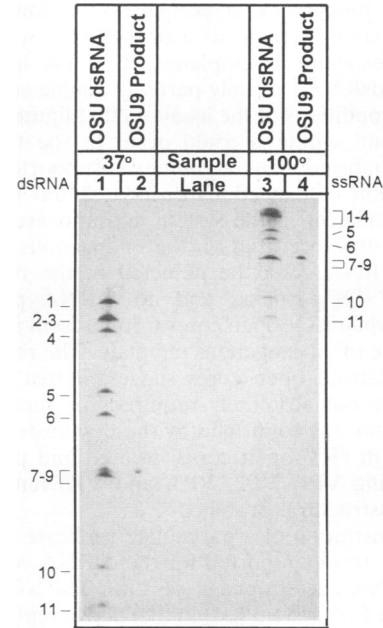


FIG. 9. In vitro replication products are unit length. The optimized in vitro replication system was programmed with the OSU9 template, and the reaction products were labeled with  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ . The products were phenol extracted and run in a denaturing gel system as described in the text.  $^{32}\text{P}$ -labeled OSU dsRNA was extracted from purified virions and run as a marker. Note that, when samples were heated to  $37^\circ\text{C}$  prior to electrophoresis, both the dsRNA marker and the OSU9 reaction product ran as dsRNA whereas, when denatured at  $100^\circ\text{C}$ , both samples ran as ssRNAs of unit length. Segment 9 (encoding VP7) of OSU is the most slowly migrating of the segments in the 7-9 complex.

be examined in these studies because the in vitro system was not template dependent and allowed runoff synthesis only of nascent negative strands that had been initiated in vivo. More recently, Gorziglia and Collins (17) described a system in which positive-sense synthetic RNAs were replicated and transcribed when transfected into cells and complemented by infection with rotavirus. This system was used to identify *cis*-acting replication signals in the 3'-terminal 19 nt of the rotavirus reporter RNA. Here, we described a template-dependent, in vitro replication system for replication of rotavirus RNA. The template dependence of this system, coupled with its ability to utilize in vitro transcripts derived from rotavirus cDNAs, will allow an analysis of *cis*-acting signals on the RNA templates that are involved in (i) binding of the template to the particulate replicase, (ii) packaging of the replication product, and (iii) initiation and synthesis of the negative-strand RNA. In addition, the demonstration that baculovirus-expressed VP1/2/3 particles exhibit replicase activity will make the various components of the particulate replicase accessible to genetic analysis of binding, packaging, and polymerase domains within the individual protein species composing the particulate replicase.

The active replicase identified here is a complex of rotavirus structural proteins VP1, VP2, and VP3 and appears to be particulate. The particulate nature of the replicase activity is consistent with the inability to obtain replicase activity from solubilized rotavirus structural proteins. The open-core form of the particulate replicase produced from native SA11-4F virions contained a slight amount of endogenous dsRNA that



appeared to function as a template at a low level in the replicase reaction, leading to a background synthesis in the absence of exogenous templates. It seems likely that the endogenous dsRNA was only partially double stranded under the low-salt conditions of the assay, so that limited initiation of negative strand synthesis could occur as the termini of the segments denatured. This argument is supported by the fact that replication of exogenous positive-strand templates was much more efficient in the system and appeared to suppress the synthesis that occurred on the endogenous template. No endogenous RNA could be detected in the baculovirus-expressed rRV SS particles, and no dsRNA products were synthesized when rRV open cores were present in the reaction in the absence of an exogenous template. The results obtained with virion-derived open cores suggested that nonstructural proteins were not absolutely required for replicase activity. This suggestion was confirmed by the demonstration of replicase activity in rRV open cores isolated and prepared from cells expressing VP1, VP2, VP3, and VP6 but none of the rotavirus nonstructural proteins.

Our demonstration of a particulate replicase in rotavirus is similar to the results reported for the dsRNA yeast virus L-A and the dsRNA bacteriophage  $\phi 6$  (36). The rotavirus system resembles the L-A system (14) in that active replicase could be generated by dialysis of native cores or rRV cores into low-ionic-strength buffer. In the  $\phi 6$  system, procapsids exhibited replicase activity (28). There is no identified equivalent of the procapsid in rotavirus, but the virion-derived open core or rRV open core is organizationally complex and contains several protein species like the procapsid of  $\phi 6$ . The rotavirus system is similar to both the L-A (13) and the  $\phi 6$  (19) systems in that the particulate replicase can be expressed *in vitro* from viral cDNAs.

We demonstrated that native viral mRNAs could serve as templates for replication of the negative strand of dsRNA. However, the synthesis of the various segments of dsRNA was not equimolar, as seen for replicase systems isolated from rotavirus-infected cells (29). We do not understand the basis of this nonequimolar synthesis of dsRNA but suggest (i) that *cis*-acting replication signals on the various template species may vary in their binding affinity for the replicase or (ii) that equimolarity of synthesis could be stimulated by the presence of viral nonstructural proteins, other viral structural proteins, or some host cell factor in the system. We also demonstrated that transcripts of viral cDNA, having precise viral 5' and 3' termini, could be replicated in the system. Both capped native viral mRNAs and uncapped transcripts could be replicated in the system, indicating that the presence of a cap was not required for replication of a template RNA. Purified rotavirus dsRNA did not function as a template. Nonrotavirus RNAs were not replicated in the system, indicating specific recognition of viral RNA by the particulate replicase.

A preliminary examination of *cis*-acting replication signals on a template RNA representing segment 9 of virus strain OSU demonstrated that the replication signals are localized on the template. Specifically, the ability of OSU9- $\Delta$ RV transcripts to be replicated indicated that no required replication signals reside within the *EcoRV* fragment extending from nt 87 to 600 of the 1,062-nt OSU segment 9. Truncation of the template by as few as 26 nt at the 3' terminus yielded reporter RNAs that could not be replicated, a finding consistent with Gorziglia and Collins's (17) finding of *cis*-acting signals in the 3'-terminal 19 nt. This indicates that *cis*-acting replication signals reside at or very near the 3' terminus, although it is not known if these signals are involved in binding the template to the replicase, the initiation of negative-strand synthesis, or both. Finally, we

do not yet know if required signals reside at the 5' terminus of the template. Binding of NS34 (NSP3) to rotavirus RNA has been mapped to sites at both 5' and 3' termini (26), and the 3'-terminal binding has been shown to be mediated by the 3'-terminal consensus sequence present on each rotavirus mRNA (32), suggesting the possibility that important signals could reside at both termini of the template. However, any signal residing at the 5' terminus must lie within the first 87 nt to be consistent with our results for the replication of OSU9- $\Delta$ RV, and the constructs examined by Gorziglia and Collins (17) require that they lie in the 5'-terminal 44 nt.

The template requirements of the rotavirus replicase reaction were similar to those in the L-A and  $\phi 6$  systems (36). Specifically, truncations at the 3' terminus of rotavirus templates resulted in an inability of the templates to replicate. This is consistent with the results obtained with L-A, in which the 3'-terminal 4 nt were critical for replication (9), although we do not know if internal sites are also required on rotavirus templates as they are on L-A templates. Similarly, *cis*-acting replication sites have been shown to reside at some distance from the 5'-terminus of  $\phi 6$  templates (18), although the proximity to the 3' terminus has not been determined (12). Unlike the situation in  $\phi 6$  (12), there does not appear to be a requirement for complete packaging of all 11 templates for activation of replicase activity in the rotavirus particulate replicase. We showed the ability of rRV open cores to replicate the single template species, OSU9. This indicates that either (i) there is no requirement for all replication sites being filled for replicase activity or (ii) the conditions of our assay are sub-optimal and relax such a requirement.

The optimized replication system presented here has several features that merit comment. (i) The rotavirus replicase appeared to have specificity for viral templates, regardless of whether  $Mg^{2+}$  or  $Mn^{2+}$  was the divalent cation. This template specificity is like that seen with the L-A virus (14). In contrast, template specificity of the  $\phi 6$  replicase was sensitive to the divalent cation, and viral and nonviral templates were replicated in the presence of  $Mn^{2+}$  but template specificity was seen in the presence of  $Mg^{2+}$  (19). Furthermore, in  $\phi 6$  the requirement for all templates to be packaged before the replicase is activated was relaxed in the presence of  $Mn^{2+}$  (12). In rotavirus, the divalent cation had no effect on the ability of the replicase to replicate a single template species in the absence of the other 10 templates. (ii) As for both the L-A (14) and the  $\phi 6$  (19) systems, the addition of polyethylene glycol to the system increased activity. The effect of polyethylene glycol appears to be to make a limiting factor(s) in the system more concentrated (27). Polyethylene glycol does not appear to act by concentrating some host factor that is present in the reaction mixture in trace amounts, as suggested for L-A. (iii) Although the rotavirus replicase clearly recognizes the templates, we do not know if the dsRNA product of the rotavirus replicase is packaged *in vitro*. The dsRNA of the L-A virus is not packed (13), and the templates of  $\phi 6$  are packaged in an ATP-dependent process prior to replication of the dsRNA which is packaged (28).

In summary, we have described a template-dependent, *in vitro* system for the replication of rotavirus dsRNA. This system consists of a particulate replicase, positive-sense RNA templates, salts, and nucleoside triphosphates. Both the template RNAs and the particulate replicase can be derived by *in vitro* expression of rotavirus cDNAs, expression of templates from transcription vectors, and expression of the replicase in cells coinfecting with four baculovirus recombinants expressing rotavirus proteins. The ability to express both the templates and the particulate replicase makes both viral components of

the system amenable to molecular genetic analyses of signals involved in the replication process. It is likely that further experiments with this system will also identify signals involved in the assortment of rotavirus genes, a process that most likely occurs at the level of recognition of RNA templates during virus assembly (34). Furthermore, it is possible that particles that have completed the replication of a complete set of rotavirus templates (cores) can be transcapsidated and rendered infectious, as can native cores (5, 6). We are currently investigating these possibilities.

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