JOURNAL OF VIROLOGY, Oct. 1990, p. 4988–4996
0022-538X/90/104988-09$02.00/0

Vol. 64, No. 10

# Rotavirus RNA Replication: VP2, but Not VP6, Is Necessary for Viral Replicase Activity

ERIC A. MANSELL AND JOHN T. PATTON*

*Department of Microbiology and Immunology, P.O. Box 016960 (R-138), University of Miami School of Medicine, Miami, Florida 33101*

Received 9 March 1990/Accepted 17 July 1990

**Temperature-sensitive mutants of simian rotavirus SA11 were previously developed and organized into 10 of a possible 11 recombination groups on the basis of genome reassortment studies. Two of these mutants, *ts*F and *ts*G, map to genes encoding VP2 (segment 2) and VP6 (segment 6), respectively. To gain insight into the role of these proteins in genome replication, MA104 cells were infected with *ts*F or *ts*G and then maintained at permissive temperature (31°C) until 9 h postinfection, when some cells were shifted to nonpermissive temperature (39°C). Subviral particles (SVPs) were recovered from the infected cells at 10.5 and 12 h postinfection and assayed for associated replicase activity in a cell-free system shown previously to support rotavirus genome replication in vitro. The results showed that the level of replicase activity associated with *ts*F SVPs from cells shifted to nonpermissive temperature was ca. 20-fold less than that associated with *ts*F SVPs from cells maintained at permissive temperature. In contrast, the level of replicase activity associated with *ts*G SVPs from cells maintained at nonpermissive temperature was only slightly less (twofold or less) than that associated with *ts*G SVPs from cells maintained at permissive temperature. Analysis of the structure of replicase particles from *ts*G-infected cells shifted to nonpermissive temperature showed that they were similar in size and density to virion-derived core particles and contained the major core protein VP2 but lacked the major inner shell protein VP6. Taken together, these data indicate that VP2, but not VP6, is an essential component of enzymatically active replicase particles.**

Rotaviruses are a major cause of acute gastroenteritis in a wide variety of animals, including humans (3). Like all members of the family *Reoviridae*, the genome of the rotaviruses consists of segmented double-stranded RNA (dsRNA) (for a review, see reference 7). In infectious rotavirus particles, the 11 genome segments are enclosed within two icosahedral shells of protein (24, 25). The inner shell of simian rotavirus SA11 contains a core comprised of the following three proteins: VP1 (125 kilodaltons [kDa]), VP2 (102 kDa), and VP3 (98 kDa) (1, 18). VP1 and VP3 are in low copy number in the core and share, to different degrees, sequence homology with the RNA polymerases of other RNA viruses (5, 7, 17). Given these findings and the fact that VP3 has GTP-binding activity, Fukuhara et al. (10) have proposed that VP1 serves as the rotavirus RNA polymerase and VP3 is responsible for the capping of viral transcripts. VP2 is the major protein component of the core (18) and has been shown to have binding activity for single- and double-stranded RNA (2). The major component of the inner shell, VP6 (45 kDa), surrounds the core (1, 18). Particles with only the inner shell proteins (single-shelled particles) have been isolated from infected cells and have an associated transcriptase activity able to synthesize viral mRNA in vitro (4, 15, 19). The ability of single-shelled particles to transcribe the genome is dependent on the presence of VP6, since removal of this protein from such particles with chaotropic agents yields core particles that are transcriptionally inactive (1, 30). Reconstitution of the core particles with purified VP6 restores their ability to synthesize mRNA. The outer shell of SA11 virions consists of the trypsin-sensitive hemagglutinin, VP4 (87 kDa) (9, 16), and the glycoprotein, VP7 (37 kDa) (6).

* Corresponding author.

Cell-free systems that support the synthesis of dsRNA by intracellular subviral particles (SVPs) have been used to study the molecular biology of rotavirus RNA replication (11, 15, 20–22). The results of such studies suggest that in the infected cell, viral mRNAs undergo assortment to form particles that contain 11 different plus-strand RNAs, each representing a different genome segment. Then, RNA-dependent RNA polymerases (replicases) in each particle asymmetrically synthesize viral dsRNA by using the plus-strand RNAs as templates for minus-strand synthesis (20). A feature of newly formed replicase particles is that portions of the plus-strand RNA templates extend from their surfaces (22). During replication, the plus-strand templates are drawn into the replicase particle where they are converted to dsRNA. Following synthesis, the dsRNA product is not released but remains associated with the particle in which it is made (20). Thus, replicase particles serve as direct precursors of virions. In previous studies, we provided evidence indicating that morphogenesis of the rotavirus single-shelled particle probably occurs concurrently with RNA replication and that VP6 may not be required for replicase activity (11). Our results suggested that single-shelled particles were formed by the sequential addition of VP2 and VP6 to protein complexes containing VP1 and VP3 as well as several nonstructural proteins (NS35, NS34, and NS26).

Temperature-sensitive (*ts*) mutants of rotavirus SA11 were previously developed and organized into 10 of a possible 11 recombination groups on the basis of genome reassortment studies (12, 13, 26, 27). Two of these mutants, *ts*F and *ts*G, map to genes encoding VP2 (segment 2) and VP6 (segment 6), respectively (13). To gain insight into the role of VP2 and VP6 in genome replication, we examined SVPs recovered from *ts*F- and *ts*G-infected cells shifted from permissive (31°C) to nonpermissive temperature (39°C) late in infection for associated replicase activity. Our results

indicate that shift up to nonpermissive temperature caused at least a 20-fold decrease in the levels of replicase activity in *ts*F-infected cells. In contrast, shift up to nonpermissive temperature caused only a slight decrease (twofold or less) in the levels of replicase activity in *ts*G-infected cells. Electrophoretic analysis of replicase particles from *ts*G-infected cells shifted to nonpermissive temperature showed that they were similar in structure to virion-derived cores, containing the major core protein VP2 but lacking the major inner shell protein VP6. These studies demonstrate that VP2, but not VP6, is required for rotavirus genome replication in vitro. From these data, we conclude that the viral replicase and transcriptase differ in their minimal protein requirement for enzyme activity.

## MATERIALS AND METHODS

**Cells and virus.** Fetal rhesus monkey kidney (MA104) cells were cultured in Eagle minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Stocks of wild-type (wt) simian rotavirus SA11 were prepared from plaque-purified virus, as described elsewhere (20). The SA11 *ts* mutants, *ts*F(2124) and *ts*G(2130), were kindly provided by R. F. Ramig (Baylor College of Medicine). Stocks of *ts*F and *ts*G were prepared like that of wt virus, except *ts* virus was allowed to adsorb at permissive temperature (31°C) and *ts*-infected cells were maintained at 31°C until harvested. Stocks of *ts*F and *ts*G used as an inoculum represented the third passage from triply plaque-purified virus. Virus was activated by incubation for 30 min at 37°C in the presence of 5 μg of trypsin (1:250; Difco Laboratories) per ml immediately prior to infection.

**Preparation of SVPs.** Confluent monolayers of MA104 cells in 100-mm tissue culture dishes were infected with 5 to 15 PFU of trypsin-activated rotavirus per cell. Virus was allowed to adsorb to cells for 1 h at 31°C before the inoculum was removed and replaced with serum-free MEM containing 5 μg of dactinomycin (actinomycin D) per ml. Infected cell cultures were then maintained at 31°C until 9 h postinfection (p.i.), when some cultures were shifted to 39°C. SVPs were prepared at 9 h p.i. from cultures maintained at 31°C and at 10.5 and 12 h p.i. from cultures maintained in parallel at 31 and 39°C. For harvesting, infected cells were scraped into hypotonic buffer (3 mM Tris hydrochloride [pH 8.1], 0.5 mM $MgCl_2$, 3 mM NaCl), incubated on ice for 10 min, and then disrupted by Dounce homogenization as described previously (15). Nuclei and large cellular debris were removed from lysates by centrifugation at 12,000 × *g* for 10 min. The cleared lysates were layered onto 3-ml 15 to 30% sucrose gradients (wt/wt) in TMN buffer (3 mM Tris hydrochloride [pH 8.1], 66 mM $NH_4Cl$, 3 mM magnesium acetate, 14 mM potassium acetate, 1 mM dithioerythritol). The gradients were centrifuged at 200,000 × *g* for 2 h at 4°C. The pellets were suspended in HGD buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] hydrochloride [pH 7.6], 10% glycerol, 2 mM dithioerythritol) and used as the source of SVPs in the cell-free system (15). Approximately 1 μl of SVP suspension was obtained from 6 × $10^5$ infected cells.

$^{35}$S-labeled SVPs were prepared from infected MA104 cells maintained in methionine-free MEM containing 10 μCi of $^{35}$S-amino acids (Tran$^{35}$S-label, 1,049 Ci/mmol; ICN Pharmaceuticals Inc.) per ml for 30 min immediately prior to harvest. Cells were incubated in methionine-free MEM for 1 h directly before the addition of label.

**Cell-free assay of SVPs for RNA polymerase activity.** SVPs were assayed for the presence of associated replicase activity by using a cell-free system previously shown to support the synthesis of genome-length dsRNA in vitro (20). The components of reaction mixtures (25 μl) were as described before (11) and included 10% SVP suspension (by volume), 20 μCi of [$^{32}$P]UTP (600 Ci/mmol; ICN), and 5 μM UTP. Unless otherwise noted, reaction mixtures were incubated for 90 min at 30°C. The RNA products of some reactions were recovered by phenol extraction and ethanol precipitation.

**Cell-free assay of single-shelled particles for transcriptase activity.** Single-shelled particles were purified from lysates of infected cells by isopycnic centrifugation on CsCl gradients (23). Transcription reaction mixtures (50 μl) contained ca. 0.015 $A_{280}$ units of single-shelled particles; 120 mM Tris hydrochloride, pH 8.5; 8 mM $MgCl_2$; 40 mM NaCl; 0.1 mM S-adenosylmethionine; 2 mM [each] ATP, GTP, CTP, and UTP; and 0.6 mg of bentonite and were incubated for 30 min (30). Bentonite was removed from reaction mixtures by brief centrifugation (30 s at 6,000 × *g*), and RNA products were recovered by phenol-chloroform extraction and ethanol precipitation.

**Electrophoretic analysis of SVPs, RNAs, and proteins.** To analyze SVPs present in the cell-free system, 12.5-μl portions of reaction mixtures were brought to a volume of 25 μl with TMN buffer, treated with 1 mg of RNase A per ml for 20 min at 20°C, and then adjusted to 1% CHAPS (Sigma Chemical Co.). SVPs in the samples were resolved by electrophoresis at 70 V for 15 to 18 h at room temperature on 0.6% agarose gels containing 50 mM Tris-glycine, pH 8.8 (TGA gels) (11). Except in cases in which RNAs or proteins were to be eluted, TGA gels were dried prior to autoradiography. To recover SVPs, RNA, or protein from TGA gels, appropriate regions were cut out of the gel, homogenized by passage through a syringe (without a needle), and then passed through a syringe plugged with glass wool to remove residual agarose. Prior to further analysis, RNA recovered from gels was deproteinized by phenol extraction and ethanol precipitation.

$^{32}$P-labeled dsRNAs were analyzed by electrophoresis on 10% polyacrylamide gels and by autoradiography (23). $^{32}$P-labeled mRNAs synthesized in vitro by single-shelled particles were resolved by electrophoresis on 4% polyacrylamide gels containing 7 M urea (30). $^{35}$S-labeled protein was analyzed by electrophoresis on 12% polyacrylamide gels and fluorography (20).

**Densitometric analysis of dsRNA product.** To quantitate the amount of dsRNA produced by various preparations of SVPs in the cell-free system, autoradiographs of the $^{32}$P-labeled dsRNA products of reactions resolved on polyacrylamide gels were analyzed by using a scanning densitometer (model GS300; Hoefer Scientific Instruments) integrated to a MacIntosh SE computer. Band intensities were integrated by the GS-370 Data System (Hoefer Scientific Instruments). The integration values used for this study represented the average intensities obtained from two independent scans of each of the bands analyzed.

**Preparation of single- and double-shelled virus and virion-derived core particles.** $^{35}$S-labeled single- and double-shelled virus was prepared from cells continuously labeled with 10 μCi of $^{35}$S-amino acids (Tran$^{35}$S-labeled, 1,049 Ci/mmol; ICN) per ml. Virus was purified by centrifugation at 120,000 × *g* on CsCl gradients (23). Cores were prepared by treating single-shelled SA11 virus with $CaCl_2$ (2).

## RESULTS

**Effect of shift up to nonpermissive temperature on the levels of replicase activity in *ts*F- and *ts*G-infected cells.** The results of previous studies have indicated that the essential protein components of rotavirus replicase and transcriptase particles may differ. Whereas VP2 and VP6 appear to be necessary components of transcriptase particles (1, 30), doubt exists as to whether these same proteins are also necessary components of replicase particles (11, 15). In this study, we have used *ts* mutants of simian rotavirus SA11 that map to the viral genes encoding VP2 (*ts*F) and VP6 (*ts*G) as a means of determining whether replicase particles require these structural proteins for polymerase activity.

To examine the role of VP2 and VP6 in rotavirus RNA replication, MA104 cells were infected with wt virus or with a *ts* mutant, *ts*F or *ts*G. The cells were then maintained at 31°C until 9 h p.i. when some cells were shifted to 39°C, a temperature nonpermissive for growth of the *ts* mutants. To analyze the effect of temperature shift up on the levels of replicase particles in cells infected with wt and mutant viruses, SVPs were prepared at 10.5 and 12 h p.i. both from cells shifted to 39°C at 9 h p.i. and from cells maintained continuously at 31°C. The SVPs were then assayed for RNA polymerase activity in a cell-free system that supports rotavirus RNA replication and transcription in vitro. $^{32}$P[UTP] was included in reactions to radiolabel the RNA products of the system. The products of reactions were analyzed for the presence of genome-length $^{32}$P-labeled dsRNA by electrophoresis on 10% polyacrylamide gels and by autoradiography. In this gel system, genome-length dsRNAs can be fully resolved, while full-length viral mRNAs migrate closely together at a position between the origin of the gel and the dsRNA of genome segment 1 (20). As a quantitative measure of the level of replicase activity associated with SVPs, the intensities of bands representing $^{32}$P-labeled dsRNAs on autoradiographs were determined by densitometry.

Electrophoretic analysis of $^{32}$P-labeled RNAs synthesized in the cell-free system showed that SVPs recovered at 10.5 and 12 h p.i. from wt-infected cells (wt SVPs) either maintained at 31°C throughout infection or shifted to 39°C at 9 h p.i. contained particles with associated replicase activity (Fig. 1). The level of replicase activity associated with wt SVPs from cells shifted to 39°C was less than twofold greater at 10.5 h p.i. and was three- to fourfold greater at 12 h p.i. than that of wt SVPs maintained continuously at 31°C (Table 1). Electrophoretic analysis of $^{32}$P-labeled RNAs made in the cell-free system showed that SVPs prepared at 9, 10.5, and 12 h p.i. from *ts*F-infected cells (*ts*F SVPs) maintained at 31°C also contained particles with replicase activity (Fig. 1). In comparison, *ts*F SVPs prepared at 10.5 and 12 h p.i. from cells shifted to 39°C at 9 h p.i. synthesized no or little dsRNA in the cell-free system (Fig. 1). In fact, densitometric analysis revealed that the overall level of $^{32}$P-labeled dsRNA made by *ts*F SVPs prepared 1.5 and 3 h after shift up was less than 5% of that made by *ts*F SVPs from cells maintained continuously at 31°C (Table 1). Residual levels of replicase activity which remain associated with *ts*F SVPs from cells shifted to 39°C may be due to the inherent "leakiness" of the mutant even at nonpermissive temperature. In summary, these results indicated that *ts*F-infected cells shifted from permissive to nonpermissive temperature lost the ability to produce functional replicase particles. Hence, since the temperature-sensitive lesion of *ts*F maps to genome segment 2, the protein product of this segment, VP2, is likely an essential component of the viral replicase complex. Despite

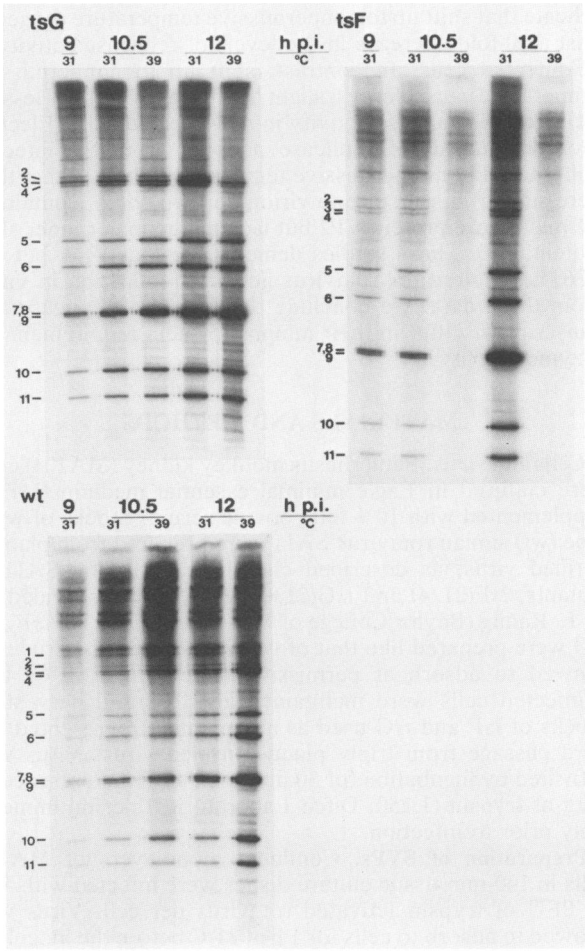


FIG. 1. Synthesis of dsRNA by replicase particles from *ts*F-, *ts*G-, and wt-infected cells. SVPs were recovered at the indicated times (h p.i.) from *ts*F-, *ts*G-, and wt-infected cells maintained throughout infection at permissive temperature (31°C) or shifted from permissive to nonpermissive temperature (39°C) at 9 h p.i. SVPs were assayed for associated polymerase activity in the cell-free system in the presence of $^{32}$P[UTP]. The $^{32}$P-labeled RNA products were recovered by phenol extraction and analyzed by electrophoresis on 10% polyacrylamide gels and autoradiography. The position of SA11 genome segments 2 to 11 in the gels are indicated. The $^{32}$P-labeled dsRNA of segment 1 was difficult to detect because of high backgrounds in the upper part of the gel caused by the nearby migration of radiolabeled viral mRNAs.

the inability of SVPs from *ts*F-infected cells shifted to 39°C to synthesize dsRNA, they retained the ability to synthesize high-molecular-weight RNAs in the cell-free system, presumably because of the presence of functional transcriptase particles.

SVPs prepared at 10.5 and 12 h p.i. from *ts*G-infected cells (*ts*G SVPs) either maintained at 31°C throughout infection or shifted to 39°C at 9 h p.i. contained readily detectable levels of associated replicase activity (Fig. 1). The level of $^{32}$P-labeled dsRNA made in the cell-free system by *ts*G SVPs prepared at 10.5 h p.i. from cells shifted to 39°C was ca. 80% of that made by *ts*G SVPs from cells maintained at 31°C (Table 1). *ts*G SVPs prepared at 12 h p.i. from cells shifted to 39°C contained replicase activity at a level that was approximately one-half of that detected for *ts*G SVPs prepared from cells maintained at 31°C (Table 1). Taken together, these

TABLE 1. Effect of temperature shift on the levels of replicase activity associated with SVPs from *ts*F- and *ts*G-infected cells

| Time of harvest (h p.i.) | Relative level of replicase activity of SVPs (% at 39°C/% at 31°C)[a] | | |
|---|---|---|---|
| | wt | *ts*G | *ts*F |
| 10.5 | 190 | 78 | <5 |
| 12 | 340 | 46 | <5 |

[a] SVPs were recovered at 10.5 and 12 h p.i. from wt-, *ts*F-, and *ts*G-infected cells maintained throughout infection at 31°C or shifted from 31 to 39°C at 9 h p.i. SVPs were assayed for RNA polymerase activity in the cell-free system in the presence of $^{32}$P[UTP]. The $^{32}$P-labeled RNA products were deproteinized by phenol extraction and analyzed by electrophoresis on 10% polyacrylamide gels and by autoradiography. The intensities of bands on the autoradiographs representing the $^{32}$P-labeled dsRNAs of segments 5, 6, 10, and 11 were determined by scanning densitometry. The average intensity for segments 5, 6, 10, and 11 of SVPs recovered from cells shifted to 39°C was divided by that for SVPs recovered from cells maintained at 31°C and multiplied by 100 (% at 39°C/% at 31°C).

results indicated that shift up from permissive to nonpermissive temperature had little short-term effect on the formation of functional replicase particles in *ts*G-infected cells. Even with long-term incubation (3 h) at nonpermissive temperature, significant levels of replicase particles continued to be produced in *ts*G-infected cells. Since the *ts* lesion of *ts*G maps to genome segment 6, these results raised the possibility that the protein product of this segment, VP6, was not an essential component of the viral replicase complex.

**Characteristics of particles with replicase activity in *ts*G- and *ts*F-infected cells.** To test whether replicase particles that lacked VP6 were able to support rotavirus genome replication, SVPs were prepared at 10.5 and 12 h p.i. from *ts*G-infected cells shifted to 39°C at 9 h p.i. and at the same times from *ts*G-infected cells maintained throughout infection at 31°C. The *ts*G SVPs were assayed for polymerase activity in the cell-free system in the presence of $^{32}$P[UTP]. Afterwards, portions of the reactions were treated with RNase A to remove single-stranded RNAs and then subjected to electrophoresis on 0.6% agarose gels containing 50 mM Tris-glycine, pH 8.8 (TGA gels). The TGA gel system was previously shown to allow the resolution of rotavirus double- and single-shelled virions and virion-derived cores (11). Most of the nuclease-resistant $^{32}$P-labeled products of reactions containing *ts*G SVPs recovered at 9, 10.5, and 12 h p.i. from cells maintained at 31°C migrated on TGA gels near the position of single-shelled virions (Fig. 2). However, some of the $^{32}$P-labeled products made by these SVPs also comigrated on TGA gels with virion-derived cores. The migration pattern of the RNA products of *ts*G SVPs maintained at 31°C on TGA gels was similar to that reported earlier for wt SA11 SVPs assayed in the cell-free system and treated with RNase prior to electrophoresis (11). Nearly all the nuclease-resistant $^{32}$P-labeled RNA made in vitro by *ts*G SVPs prepared at 10.5 and 12 h p.i. from cells shifted to 39°C comigrated on TGA gels with virion-derived cores (Fig. 2). The amount of the nuclease-resistant $^{32}$P-labeled RNA made by *ts*G SVPs from cells shifted to 39°C that comigrated with virion-derived cores was severalfold greater than that made by *ts*G SVPs from cells maintained at 31°C. This result suggested that shifting *ts*G-infected cells to nonpermissive temperature caused the accumulation of corelike particles that were able to synthesize nuclease-resistant RNA. In contrast to the $^{32}$P-labeled RNA products of *ts*G SVPs from cells maintained at 31°C, none of the RNA products of *ts*G SVPs from cells shifted to 39°C comigrated near single-shelled virions on TGA gels (Fig. 2). Considered together, these results

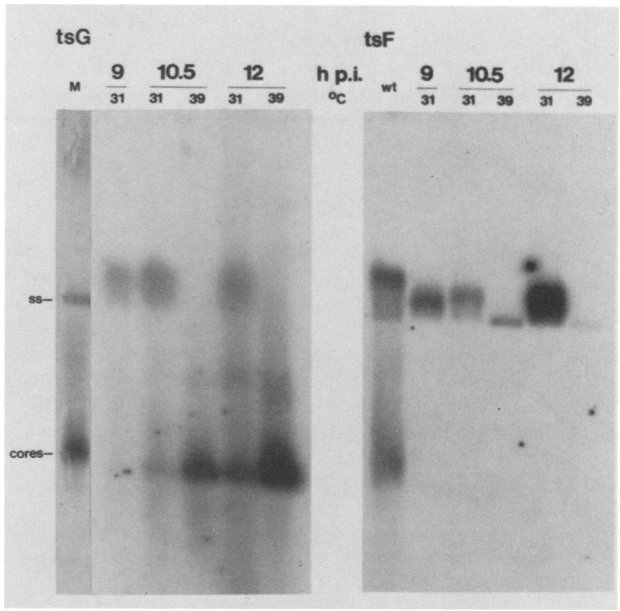


FIG. 2. Analysis of *ts* SVPs containing newly made dsRNA by electrophoresis on TGA gels. SVPs were recovered at the indicated times (h p.i.) from *ts*F- and *ts*G-infected cells maintained throughout infection at permissive temperature or shifted from permissive to nonpermissive temperature at 9 h p.i. SVPs were assayed for associated polymerase activity in the cell-free system in the presence of $^{32}$P[UTP]. Portions (12.5 μl) of the reaction mixture were treated with CHAPS and RNase and electrophoresed on TGA gels. As markers, $^{35}$S-labeled single-shelled particles (ss) and virion-derived cores were electrophoresed in a parallel lane (lane M) and detected by fluorography. SVPs recovered from wt-infected cells at 9 h p.i. from cells maintained at 31°C were electrophoresed in lane wt.

indicated that corelike replicase particles from *ts*G-infected cells shifted from permissive to nonpermissive temperature were primarily responsible for the synthesis of nuclease-resistant RNA, i.e., dsRNA, in the cell-free system. Since the corelike replicase particles comigrated in TGA gels with structures that lacked the major inner shell protein, VP6, i.e., virion-derived cores, these data also suggested that VP6 was not an essential component of the replicase complex.

The $^{32}$P-labeled products of reactions containing *ts*F SVPs were also examined by electrophoresis on TGA gels. While wt and *ts*G SVPs from cells maintained at 31°C synthesized $^{32}$P-labeled products in the cell-free system that comigrated on TGA gels with virion-derived cores, *ts*F SVPs from cells maintained at 31°C did not (Fig. 2). Instead, all the detectable products of *ts*F SVPs from cells maintained at 31°C migrated with or near single-shelled particles. This result suggests that even under permissive temperature, mutations in the *ts*F genome result in changes in the relative amounts of particles with RNA polymerase activity in the infected cell compared with cells infected with wt virus. As expected from results presented above, *ts*F SVPs from cells shifted to 39°C compared with cells kept at 31°C showed a significant decrease in ability to synthesize nuclease-resistant RNA products in the cell-free system detectable by electrophoresis on TGA gels (Fig. 2).

**Synthesis of viral dsRNA by corelike particles in *ts*G-infected cells.** To examine the possibility that corelike particles could support rotavirus RNA replication, SVPs were recovered at 12 h p.i. from *ts*G-infected cells shifted from 31

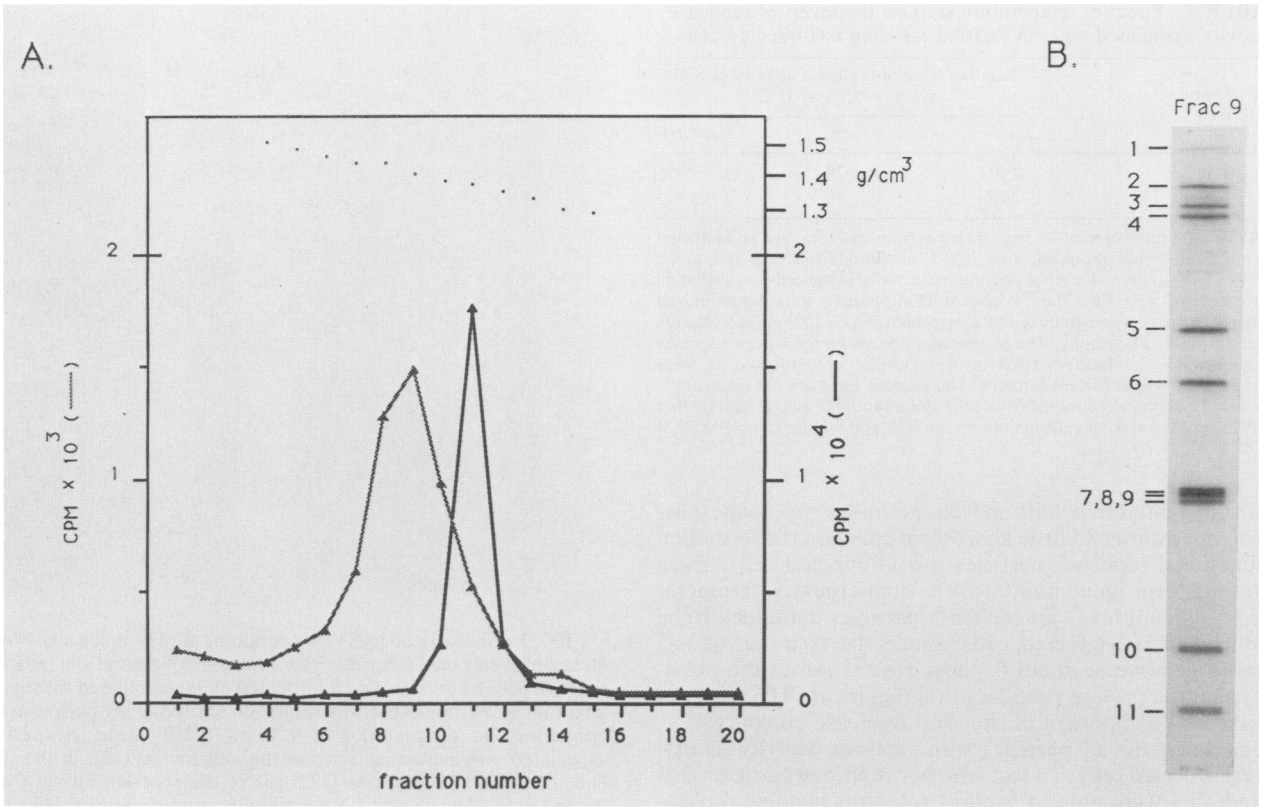


FIG. 3. Analysis of *ts*G replicase particles by CsCl centrifugation. SVPs recovered at 12 h p.i. from *ts*G-infected cells shifted from 31 to 39°C at 9 h p.i. were assayed for associated polymerase activity in the cell-free system. A portion of the reaction mixture was treated with CHAPs and RNase and electrophoresed on a TGA gel. $^{32}$P-labeled particles comigrating in the gel with virion-derived cores were eluted and layered over a 4-ml gradient of 30 to 55% (wt/wt) CsCl in TMN buffer (A, hatched line). The gradient was centrifuged for 18 h at 120,000 × *g* in a rotor (model SW50.1; Beckman Instruments, Inc.) at 4°C. $^{35}$S-labeled single-shelled virions were included in the gradient as a marker (solid line). Fractions (220 μl) of the gradient were collected, and 20-μl portions of each fraction were assayed for acid-precipitable radioactivity. Fractions were assayed for density (g/cm$^3$) by refractometry. RNA in fraction 9 of the gradient containing *ts*G corelike replicase particles (hatched line) was purified by phenol-chloroform extraction and analyzed by gel electrophoresis and autoradiography (B).

to 39°C at 9 h p.i. The SVPs were assayed for polymerase activity in the cell-free system in the presence of $^{32}$P[UTP]. Afterwards, a portion of the reaction was digested with RNase and subjected to electrophoresis on a TGA gel. $^{32}$P-labeled products that migrated on the gel to the position of virion-derived cores were recovered and centrifuged to equilibrium on gradients of 30 to 55% (wt/wt) CsCl. Analysis of the gradients showed that the $^{32}$P-labeled products banded with a peak density of ca. 1.43 g/cm$^3$ (Fig. 3). The peak density approximated that reported for the cores of bovine rotavirus (1.44 g/cm$^3$) (1). The $^{32}$P-labeled RNA that banded at 1.43 g/cm$^3$ of CsCl was deproteinized by phenol extraction and resolved by electrophoresis on a 10% polyacrylamide gel. Radiolabeled dsRNAs representing all 11 genome segments of rotavirus SA11 were present in the peak of $^{32}$P-labeled material (Fig. 3). The peak fraction also contained nearly all the $^{32}$P-labeled dsRNA recovered from the gradient. In summary, these experiments showed that particles that comigrated on TGA gels with virion-derived cores and that had a density in CsCl similar to that of virion-derived cores supported the synthesis of viral dsRNA in the cell-free system.

To determine whether the corelike replicase particles contained VP6, a protein component of single-shelled particles that is absent from virion-derived cores, SVPs were prepared at 12 h p.i. from *ts*G-infected cells that were shifted from 31 to 39°C at 9 h p.i. and that were maintained in the presence of $^{35}$S-amino acids for 30 min prior to harvest. The $^{35}$S-labeled SVPs were assayed for polymerase activity in the cell-free system in the absence of $^{32}$P[UTP]. Then, a portion of the reaction mixture was treated with RNase A and subjected to electrophoresis on a TGA gel. $^{35}$S-labeled particles in the gel that comigrated with corelike replicase particles were eluted and examined for $^{35}$S-labeled protein content by electrophoresis on a 12% polyacrylamide gel and fluorography (Fig. 4). $^{35}$S-labeled VP2 and, in some experiments, VP1 and VP3 were associated with the corelike particles eluted from the TGA gels. In contrast, little or no $^{35}$S-labeled VP6 was present in the eluted particles. These data indicated that the corelike replicase particles accumulated at nonpermissive temperature in *ts*G-infected cells that contained the major core protein VP2 but lacked the major inner shell protein VP6. In addition to $^{35}$S-labeled VP2, the nonstructural protein NS35 was found associated with the corelike replicase particles (Fig. 4). NS35 is an RNA-binding protein (unpublished results) that has been previously reported to be a component of rotavirus replication intermediates (RIs) (11, 15, 21).

**VP6 trimers in *ts*G-infected cells maintained at nonpermissive temperature.** Trimers of VP6 have been detected in rotavirus-infected cells and have been proposed to participate in the assembly of single-shelled particles in vivo (14,

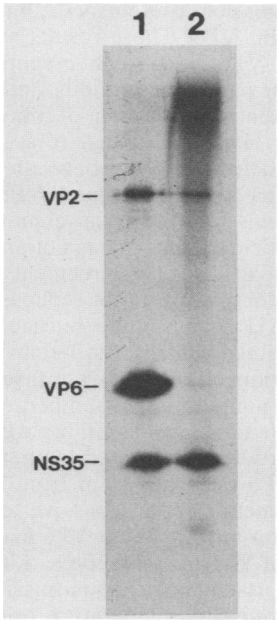


FIG. 4. Protein content of corelike replicase particles. SVPs were recovered at 12 h p.i. from *ts*G-infected cells maintained throughout infection at 31°C or from *ts*G-infected cells shifted from 31 to 39°C at 9 h p.i. All infected cells were maintained in the presence of $^{35}$S-amino acids for 30 min immediately prior to harvest. The SVPs were assayed in the cell-free system, treated with CHAPS and RNase, and electrophoresed on a TGA gel. SVPs from cells maintained at 31°C that comigrated with single-shelled virions were eluted and analyzed for $^{35}$S-labeled protein content by electrophoresis on a 12% polyacrylamide gel and fluorography (lane 1). *ts*G SVPs from cells shifted to 39°C that comigrated with virion-derived cores were similarly eluted from the TGA gel and analyzed for protein content (lane 2). Protein assignments were based on the comigration of $^{35}$S-labeled wt SVPs in the gel (data not shown).

29). The accumulation of corelike replicase particles in *ts*G-infected cells shifted to 39°C indicated that the mutant VP6 protein was defective in assembly at elevated temperature, unable to associate with core particles to form single-shelled particles. To test the possibility that VP6 was produced at nonpermissive temperature in *ts*G-infected cells but was unable to assemble into trimers, a cytoplasmic lysate was prepared at 12 h p.i. from *ts*G-infected cells shifted to 39°C at 9 h p.i. Cells were maintained in $^{35}$S-amino acids for 30 min immediately prior to harvest to label those proteins synthesized at nonpermissive temperature. All procedures used in the preparation of the cytoplasmic lysate were carried out at 39°C to avoid the possibility that the *ts*G VP6 might undergo trimerization in vitro when the temperature was lowered below nonpermissive temperature. Portions of the $^{35}$S-labeled lysates were incubated for 30 min at 37°C or for 2 min at 100°C in sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol and then subjected to electrophoresis on a 12% polyacrylamide gel and fluorography. Characteristic of VP6 trimers (8), a band of approximately 150 kDa was detected in the lane of the gel containing lysate heated to 37°C but was absent in the lane containing lysate heated to 100°C (Fig. 5). When protein in the 150-kDa band was eluted from the gel, incubated in sample buffer at 100°C, and analyzed by electrophoresis on a 12% polyacrylamide gel, the $^{35}$S-labeled material comigrated with the VP6 monomer. These results indicated that the

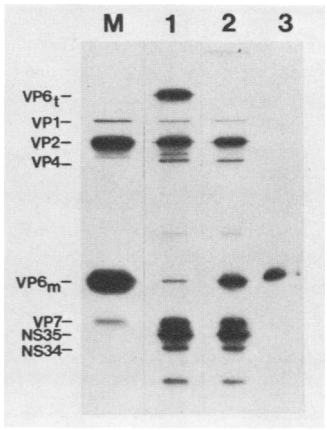


FIG. 5. VP6 trimer formation in *ts*G-infected cells at nonpermissive temperature. Cleared lysates were prepared as described in Materials and Methods at 12 h p.i. from *ts*G-infected cells shifted from 31 to 39°C at 9 h p.i. The infected cells were maintained in the presence of $^{35}$S-amino acids for 30 min prior to harvest. Procedures employed in the preparation of the cleared lysate were carried out at 39°C. Portions of the cleared lysate were incubated in sample buffer (1% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.5 M urea, 50 mM Tris hydrochloride [pH 6.8], 10% glycerol, 0.01% bromphenol blue [8]) for 30 min at 37°C (lane 1) or for 2 min at 100°C (lane 2) and examined for radiolabeled protein content by gel electrophoresis and fluorography. Material migrating at the position of the VP6 trimer ($VP6_t$) was eluted from another gel, heated to 100°C for 2 min in sample buffer, and electrophoresed in lane 3. The position of the VP6 monomer ($VP6_m$) was based on electrophoresis of $^{35}$S-labeled virions in a parallel lane (lane M).

mutant VP6 was able to assemble into trimers at nonpermissive temperature in *ts*G-infected cells.

**Effect of temperature on the in vitro synthesis of RNA by transcriptase and replicase particles from *ts*F- and *ts*G-infected cells.** To explore the possibility that replicase particles were not present in *ts*F-infected cells at nonpermissive temperature because of a *ts* defect in the assembly of VP2 into corelike particles, SVPs were purified at 12 h p.i. from *ts*F-infected cells maintained continuously at 31°C. The SVPs were assayed for associated replicase activity at incubation temperatures of 30 and 39°C in the cell-free system, and the RNA products were analyzed by gel electrophoresis. Replicase particles from *ts*F-infected cells maintained at permissive temperature were able to support the synthesis of dsRNA equally well at 30 and 39°C in vitro (Fig. 6). Thus, *ts*F replicase particles that were "preassembled" at permissive temperature were enzymatically active even when assayed at a temperature (39°C) not permissive for the formation of active replicase particles in vivo. This result suggests that the lack of replicase activity in *ts*F-infected cells at nonpermissive temperature stems from an inability of the mutant VP2 to assemble into replicase particles and is not due to the inability of preassembled replicase particles containing the mutant VP2 to synthesize dsRNA at elevated temperature.

As a final analysis of the *ts* lesion in the mutant VP2 of *ts*F and in the mutant VP6 of *ts*G, single-shelled particles were purified from *ts*F- and *ts*G-infected cells maintained at permissive temperature. Afterwards, the single-shelled particles were assayed at 30 and 45°C for associated transcriptase activity, and the RNA products were analyzed by electrophoresis on a 4% polyacrylamide gel containing 7 M urea (Fig. 6). Both *ts*F and *ts*G single-shelled particles were able

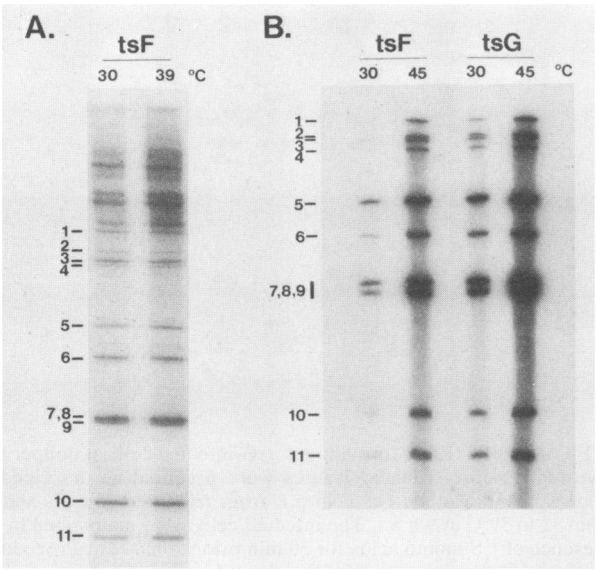


FIG. 6. Synthesis of RNA at elevated temperatures in vitro by transcriptase and replicase particles from *ts*F- and *ts*G-infected cells. (A) SVPs were purified at 12 h p.i. from *ts*F-infected cells and assayed for associated RNA polymerase activity at 30 and 39°C in reactions containing [$^{32}$P]UTP. The RNA products were recovered by phenol-chloroform extraction and analyzed by electrophoresis on a 10% polyacrylamide gel and autoradiography. (B) Single-shelled particles were recovered from lysates of *ts*F- and *ts*G-infected cells by centrifugation on CsCl gradients. Purified single-shelled particles were assayed for associated transcriptase activity at 30 and 45°C in reactions containing $^{32}$P[UTP]. The products of the reactions were purified by phenol-chloroform extraction and analyzed by electrophoresis on 4% polyacrylamide gels containing 7 M urea (30).

to direct the synthesis of viral mRNA at 45°C at levels severalfold higher than that achieved by the same particles assayed at 30°C. These results provided evidence that once assembled into single-shelled particles, the mutant VP2 and VP6 were able to function in RNA transcription even at high temperature.

## DISCUSSION

The *ts* mutants, *ts*F and *ts*G, of simian rotavirus SA11 have been mapped to the genome segments encoding the major core protein, VP2 (segment 2), and the major inner shell protein, VP6 (segment 6), respectively (13). In the study described here, we have used *ts*F and *ts*G to determine whether VP2 and VP6 are essential components of enzymatically active replicase particles. The results demonstrated that SVPs recovered from *ts*F-infected cells shifted from permissive to nonpermissive temperature contained few particles with associated replicase activity. This finding indicated that the *ts* gene product of *ts*F, VP2, is required for the formation of active replicase particles in rotavirus-infected cells. In contrast, SVPs from *ts*G-infected cells continued to exhibit high levels of associated replicase activity for at least several hours following shift up from permissive to nonpermissive temperature. At nonpermissive temperature, corelike particles accumulated in *ts*G-infected cells that, via an associated replicase activity, were able to direct the synthesis of rotavirus dsRNAs in vitro. Analysis of the corelike replicase particles showed that they had a density in CsCl similar to that of virion-derived cores and contained the major core protein, VP2, but lacked the major inner shell protein, VP6. These results showed that the *ts* product of *ts*G, VP6, is not an essential component of rotavirus replicase particles. Previous studies have indicated that VP6 is an essential component of rotavirus transcriptase particles (1, 30). Hence, although rotavirus replicase and transcriptase particles probably share a requirement for the structural proteins VP1, VP2, and VP3 for polymerase activity, VP6 seems to be only an essential component of transcriptase particles. That VP6 is not an essential component of replicase particles is in agreement with the results of an earlier study that showed that replicase particles which contained the SA11 core proteins but lacked VP6 could be isolated from infected cells by CsCl centrifugation (15).

The accumulation of corelike replicase particles in *ts*G-infected cells at nonpermissive temperature indicates that the mutant VP6 is unable to assemble with viral cores into single-shelled particles at elevated temperature. The defect in the mutant VP6 does not stem from a inability to self assemble into trimers at nonpermissive temperature. Thus, the actual *ts* lesion in the mutant VP6 may exist within the binding domain of VP6 for viral cores. Although the mutant VP6 is unable to assemble at nonpermissive temperature, if assembled at permissive temperature prior to exposure at elevated temperature, *ts*G VP6 appears to function normally. As evidence for this, single-shelled particles purified from *ts*G-infected cells maintained at permissive temperature are able to synthesize viral mRNAs in cell-free reaction mixtures incubated at 45°C. The following two lines of evidence indicate that the mutant VP2 of *ts*F also functions normally if allowed to assemble at permissive temperature prior to exposure to high temperature: (i) single-shelled particles purified from *ts*F-infected cells are able to support the synthesis of viral mRNA at 45°C, and (ii) SVPs prepared from *ts*F-infected cells maintained at permissive temperature support the synthesis of dsRNA equally well in vitro at 30 and 39°C. On the other hand, SVPs from *ts*F-infected cells maintained at nonpermissive temperature do not support the synthesis of dsRNA even when assayed in vitro at permissive temperature (30°C). Considered together, these data indicate that the mutant VP2 is not being assembled into SVPs at nonpermissive temperature. Otherwise, we would predict that particles which were assembled in vivo at 39°C by using the mutant VP2 would have had associated replicase activity when assayed in the cell-free system at 30°C. Our conclusion that mutant VP2 is defective in assembly at nonpermissive temperature is in agreement with the results of a study by Ramig and Petrie (28) which showed by electron microscopy that morphogenic intermediates are not assembled at nonpermissive temperature in *ts*F-infected cells.

The phenotypes of the mutants *ts*F and *ts*G have both been reported as negative for dsRNA (28). Our results are consistent with these findings. In the case of *ts*F, the inability of replicase particles to form at nonpermissive temperature because of a *ts* lesion in VP2 would prevent the synthesis of dsRNA in *ts*F-infected cells maintained at elevated temperature. At nonpermissive temperature in *ts*G-infected cells, while replicase particles can be formed, transcriptase particles cannot because of the inability of VP6 to assemble into single-shelled particles. Thus, the level of mRNA synthesis remains low in *ts*G-infected cells because of an inability to amplify the number of transcriptase particles. Because the formation of replicase particles is dependent on the availability of viral mRNAs to serve as templates for genome replication, the number of replicase particles that

can be formed in *ts*G-infected cells at elevated temperature must remain low. As a result, the phenotype of *ts*G-infected cells maintained continuously at high temperature would appear to be dsRNA negative.

The level of replicase activity associated with SVPs recovered at 12 h p.i. from *ts*G-infected cells shifted to nonpermissive temperature at 9 h p.i. was approximately 50% of that associated with SVPs from *ts*G-infected cells maintained at permissive temperature throughout infection (Table 1). One explanation for this decrease in activity may be that some single-shelled particles present in the *ts*G-infected cells at the time of temperature shift up are subsequently lost due to their morphogenesis into double-shelled particles. At nonpermissive temperature in *ts*G-infected cells, new single-shelled particles cannot be formed to replace any lost due to morphogenesis, because of the assembly defect in the mutant VP6 protein. Since single-shelled particles function as transcriptase particles (15), the loss of single-shelled particles from infected cells can be expected to result in a corresponding decrease in the level of viral transcription in vivo. And since viral mRNAs are essential components of replicase particles serving as the templates for dsRNA synthesis (20), a decrease in viral transcription in *ts*G-infected cells following temperature shift up could inhibit the formation of replicase complexes by limiting the size of the intracellular pool of viral mRNA. Hence, the 50% decrease in the level of replicase activity associated with SVPs recovered at 12 h p.i. from *ts*G-infected cells shifted to nonpermissive temperature may reflect the loss of single-shelled particles. In a previous study, SVPs from SA11-infected cells were assayed for polymerase activity in the cell-free system and then resolved by electrophoresis on TGA gels (11). SVPs from the system that contained newly made dsRNA migrated in the gels to positions between virion-derived cores and slightly above single-shelled particles. As an indication of the protein composition of SVPs that contained newly made dsRNA, nonassayed $^{35}$S-labeled SVPs were electrophoresed on TGA gels in parallel with the products of the cell-free system. $^{35}$S-labeled SVPs that comigrated with particles from the cell-free system that contained newly made dsRNA were eluted and examined for protein content by polyacrylamide gel electrophoresis. The results of the study suggested that the following three distinct types of SVPs were able to synthesize dsRNA in the cell-free system: (i) the single-shelled RI containing the structural proteins VP1, VP2, VP3, and VP6; (ii) the core RI containing VP1, VP2, and VP3; and (iii) the precore RI containing VP1 and VP3 (11). From this study and in contrast to the results reported here with *ts*F and *ts*G, it was proposed that neither VP6 nor VP2 was an essential component of enzymatically active replicase particles (11). However, more recent studies have demonstrated that as replicase particles synthesize dsRNA, they undergo a decrease in overall size (21). This is due to the movement of the plus-strand RNA template for replication into the replicase particle as it synthesizes dsRNA. Thus, nonassayed $^{35}$S-labeled SVPs eluted from TGA gels may not accurately reflect the protein composition of assayed replicase particles which contain newly made dsRNA and comigrate in a parallel lane of the same gel. Indeed, when $^{35}$S-labeled SVPs are assayed for polymerase activity in the cell-free system prior to electrophoresis on TGA gels, particles containing VP2 comigrate with virion-derived cores (unpublished results) to a position of the gel previously reported to contain only particles made up of VP1 and VP3 but that lacked VP2, i.e., the precore RI (11).

From our studies on the structure and function of rotavirus replicase particles, we conclude that the precore RI, a complex consisting of VP1, VP3, and viral mRNA (11), associates with VP2, resulting in the formation of the corelike replicase particle. Although either VP1 or VP3 probably serves as the viral RNA polymerase, the precore RI lacks replicase activity because of the absence of VP2. Once formed, the corelike replicase particle initiates the synthesis of rotavirus dsRNA in a manner that provides for the sequential completion of replication of the 11 genome segments (22). As RNA replication proceeds, the corelike replicase particle concurrently undergoes morphogenesis, associating with VP6 to form the single-shelled replicase particle (single-shelled RI). Following replication, the single-shelled RI serves as the precursor of double-shelled virions or functions as a transcriptase particle to synthesize viral mRNA.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI21478 from the National Institutes of Health.

We are grateful to Frank Ramig for providing the *ts* mutants and unpublished information with regard to their replication.

## LITERATURE CITED

1. **Bican, P., J. Cohen, A. Charpilienne, and R. Scherrer.** 1982. Purification and characterization of bovine rotavirus cores. J. Virol. **43**:1113–1117.
2. **Boyle, J. F., and K. V. Holmes.** 1986. RNA-binding proteins of bovine rotavirus. J. Virol. **51**:561–568.
3. **Christensen, M. L.** 1989. Human viral gastroenteritis. Clin. Microbiol. Rev. **2**:51–89.
4. **Cohen, J.** 1977. Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. **36**:395–402.
5. **Cohen, J., A. Charpilienne, S. Chilmonczyk, and M. K. Estes.** 1989. Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology **171**: 131–140.
6. **Ericson, B. L., D. Y. Graham, B. B. Mason, and M. K. Estes.** 1982. Identification, synthesis, and modifications of simian rotavirus SA11 polypeptides in infected cells. J. Virol. **42**:825–839.
7. **Estes, M. K., and J. Cohen.** 1989. Rotavirus gene structure and function. Microbiol. Rev. **53**:410–449.
8. **Estes, M. K., S. E. Crawford, M. E. Penaranda, B. L. Petrie, J. W. Burns, W.-K. Chan, B. Ericson, G. E. Smith, and M. D. Summers.** 1987. Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. J. Virol. **61**:1488–1494.
9. **Estes, M. K., D. Y. Graham, and B. B. Mason.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. **39**:879–888.
10. **Fukuhara, N., K. Nishikawa, M. Gorziglia, and A. Z. Kapikian.** 1989. Nucleotide sequence of gene segment 1 of a porcine rotavirus strain. Virology **173**:743–749.
11. **Gallegos, C. O., and J. T. Patton.** 1989. Characterization of rotavirus replication intermediates: a model for the assembly of single-shelled particles. Virology **172**:616–627.
12. **Gombold, J. L., M. K. Estes, and R. F. Ramig.** 1985. Assignment of simian rotavirus SA11 temperature-sensitive mutant groups A and E to genome segments. Virology **143**:309–320.
13. **Gombold, J. L., and R. F. Ramig.** 1987. Assignment of simian rotavirus SA11 temperature-sensitive mutant groups A, C, F, and G to genome segments. Virology **161**:463–473.
14. **Gorziglia, M., C. Larrea, F. Liprandi, and J. Esparza.** 1985. Biochemical evidence for the oligomeric (possibly trimeric) structure of the major inner capsid polypeptide (45K) of rotaviruses. J. Gen. Virol. **66**:1889–1900.
15. **Helmberger-Jones, M., and J. T. Patton.** 1986. Characterization of subviral particles in cells infected with simian rotavirus SA11.

Virology **155**:655–665.
16. **Kalica, A. R., J. U. Flores, and H. B. Greenberg.** 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque-formation. Virology **125**:194–205.
17. **Liu, M., and M. K. Estes.** 1989. Nucleotide sequence of the simian rotavirus SA11 genome segment 3. Nucleic Acids Res. **17**:7991.
18. **Liu, M., P. A. Offit, and M. K. Estes.** 1987. Identification of the simian rotavirus SA11 genome segment 3 product. Virology **163**:26–32.
19. **Mason, B. B., D. Y. Graham, and M. K. Estes.** 1983. In vitro transcription and translation of simian rotavirus SA11 gene products. J. Virol. **33**:1111–1121.
20. **Patton, J. T.** 1986. Synthesis of simian rotavirus SA11 double-stranded RNA in a cell-free system. Virus Res. **6**:217–233.
21. **Patton, J. T., and C. O. Gallegos.** 1988. Structure and protein composition of the rotavirus replicase particle. Virology **166**: 358–365.
22. **Patton, J. T., and C. O. Gallegos.** 1990. Rotavirus RNA replication: single-stranded RNA extends from the replicase particle. J. Gen. Virol. **71**:1087–1094.
23. **Patton, J. T., and S. Stacy-Phipps.** 1986. Electrophoretic separation of the plus and minus strands of rotavirus double-stranded RNAs. J. Virol. Methods **13**:185–190.
24. **Petrie, B. L., D. Y. Graham, and M. K. Estes.** 1981. Identification of rotavirus particle types. Intervirology **16**:2–28.
25. **Prasad, B. V. V., G. J. Wang, J. P. M. Clerx, and W. Chiu.** 1988. Three-dimensional structure of rotavirus. J. Mol. Biol. **199**:269–275.
26. **Ramig, R. F.** 1982. Isolation and genetic characterization of temperature-sensitive mutants of simian rotavirus SA11. Virology **120**:93–105.
27. **Ramig, R. F.** 1983. Isolation and genetic characterization of temperature-sensitive mutants that define five additional recombination groups in simian rotavirus SA11. Virology **130**:464–473.
28. **Ramig, R. F., and B. L. Petrie.** 1984. Characterization of temperature-sensitive mutants of simian rotavirus SA11: protein synthesis and morphogenesis. J. Virol. **49**:665–673.
29. **Sabara, M. K., K. F. M. Ready, P. J. Frenchick, and L. A. Babiuk.** 1987. Biochemical evidence for the oligomeric arrangement of bovine rotavirus nucleocapsid protein and its possible significance in the immunogenicity of this protein. J. Gen. Virol. **68**:123–133.
30. **Sandino, A. M., M. Jashes, G. Faúndez, and E. Spencer.** 1986. Role of the inner protein capsid on in vitro human rotavirus transcription. J. Virol. **60**:797–802.