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# Characterization and Replicase Activity of Double-Layered and Single-Layered Rotavirus-Like Particles Expressed from Baculovirus Recombinants

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**Rotavirus has a capsid composed of three concentric protein layers. We coexpressed various combinations of the rotavirus structural proteins of single-layered (core) and double-layered (single-shelled) capsids from baculovirus vectors in insect cells and determined the ability of the various combinations to assemble into viruslike particles (VLPs). VLPs were purified by centrifugation, their structure was examined by negative-stain electron microscopy, their protein content was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and GTP binding assays, and their ability to support synthesis of negative-strand RNAs on positive-sense template RNAs was determined in an in vitro replication system. Coexpression of all possible combinations of VP1, VP2, VP3, and VP6, the proteins of double-layered capsids, resulted in the formation of VP1/2/3/6, VP1/2/6, VP2/3/6, and VP2/6 double-layered VLPs. These VLPs had the structural characteristics of empty rotavirus double-layered particles and contained the indicated protein species. Only VP1/2/3/6 and VP1/2/6 particles supported RNA replication. Coexpression of all possible combinations of VP1, VP2, and VP3, the proteins of single-layered capsids, resulted in the formation of VP1/2/3, VP1/2, VP2/3, and VP2 single-layered VLPs. These VLPs had the structural characteristics of empty single-layered rotavirus particles and contained the indicated protein species. Only VP1/2/3 and VP1/2 VLPs supported RNA replication. We conclude that (i) the assembly of VP1 and VP3 into VLPs requires the presence of VP2, (ii) the role of VP2 in the assembly of VP1 and VP3 and in replicase activity is most likely structural, (iii) VP1 is required and VP3 is not required for replicase activity of VLPs, and (iv) VP1/2 VLPs constitute the minimal replicase particle in the in vitro replication system.**

Rotavirus, a member of the family *Reoviridae*, is the most important cause of severe viral gastroenteritis in humans and animals (19). The biochemical and structural properties of rotavirus particles have been reviewed elsewhere (1, 11–13, 25, 33). The relevant information for the studies reported here is outlined below. Biochemically, rotavirus virions consist of six structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and 11 segments of double-stranded RNA (dsRNA). Morphologically, virus particles consist of three concentric proteinaceous capsid layers. The innermost protein layer consists of 120 VP2 molecules (7a, 32). VP1, VP3, and the 11 segments of genomic dsRNA are internal to the VP2 layer and have been proposed to be organized into a subcore (31). VP1 and VP3 are present in a few copies per virion. VP1 sequences share the common motifs of all RNA polymerases (9, 30), and VP1 in rotavirus double-layered particles can be cross-linked to azido-ATP, causing a corresponding decrease in the ability of particles to synthesize mRNA (38). VP3 binds GTP specifically and is assumed to be a guanylyltransferase (23, 29). Core-like particles containing VP1, VP2, and VP3 derived from either virions or baculovirus coexpression of VP1/2/3/6 have recently been shown to direct the synthesis of dsRNA, synthesizing negative-strand RNA on positive-strand RNA templates (8), an enzymatic activity called the viral replicase. Temperature-sensitive mutants have been used to demonstrate a role for VP2 in viral replicase activity (24). The middle protein layer is composed of 780 molecules of VP6 which are present in the form of 260 trimers and constitute the most abundant protein component of the virion. Temperature-sensitive mutants have been used to show that VP6 is not necessary for replicase activity (24). The addition of VP6 to cores activates the particle-associated RNA-dependent RNA polymerase (transcriptase), which synthesizes viral mRNA, and suppresses replicase activity (20). Thus, VP6 plays a role in modulating the enzymatic activity of single- and double-layered particles (20). The outermost protein layer is composed of 120 molecules of VP4 and 780 molecules of glycoprotein VP7. Addition of the outer capsid suppresses the activity of the viral transcriptase and results in the formation of a mature virus particle.

Each of the rotavirus genes encoding one of the six structural proteins has been cloned into the baculovirus expression system and expressed in insect cells. VP6 alone is capable of forming VP6 oligomers (14), and VP2 alone assembles into VP2 viruslike particles (VLPs) (21, 39). Coexpression of VP2 and VP6 results in the formation of VP2/6 double-layered VLPs (21). Coinfection of cells with different combinations of genes encoding the major rotavirus structural proteins of the triple-layered virus particle (VP2, VP4, VP6, and VP7) results in the formation of VLPs with different protein compositions, and these VLPs maintain the structural and functional characteristics of their cognate natural particles (10).

Here, we describe the coexpression of VLPs and the complexes of the innermost two layers of the rotavirus capsid, including VP2, the major core protein, VP6, the protein of the middle layer, and VP1 and VP3, the minor protein species

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present in the subcore. The various VLPs and complexes were characterized morphologically and for protein content and replicase activity.

## MATERIALS AND METHODS

**Cells and virus.** *Spodoptera frugiperda* (Sf9) cells were grown and maintained in TNM-FH medium containing 10% fetal bovine serum (FBS) (36). Baculovirus recombinants encoding the following rotavirus proteins were used: VP1 of virus strain RF (pVL941/Rf-1) (9), VP2 of virus strain RF (BacRf2A) (21), VP3 of virus strain SA11 (pVL1393/SA11-3) (23), and VP6 of virus strain SA11 (pAc461/SA11-6) (14).

Simian rotavirus strain SA11-4F was cultivated in fetal rhesus monkey kidney (MA104) cells in the presence of trypsin and was purified as previously described (5, 15). Double-layered particles (previously called single-shelled particles) were obtained from purified triple-layered particles by treatment with EDTA followed by CsCl density gradient centrifugation (3, 6). Single-layered particles (cores) were obtained from purified double-layered particles by treatment with $CaCl_2$ followed by CsCl density gradient centrifugation (3, 7).

**Production and purification of rotavirus double-layered VLPs.** Double-layered VLPs composed of VP1/2/3/6, VP2/3/6, VP1/2/6, and VP2/6 were prepared essentially as described by Crawford et al. (10). Briefly, $6 \times 10^8$ Sf9 cells in ~50 ml of TNM-FH–10% FBS medium were inoculated with desired combinations of the baculovirus recombinants at a multiplicity of infection of 5 PFU per cell for each recombinant and were incubated for 2 h for adsorption. The inoculum was removed by low-speed centrifugation, and the infected cell pellet was resuspended in 220 ml of Grace's insect medium containing 0.5% FBS. The infected cells were maintained in spinner flasks. The protease inhibitors aprotinin and leupeptin (0.5 μg/ml each) were added daily from days 2 through 5 postinfection. The infected cells were harvested 6 days postinfection.

Double-layered VLPs were stable and were released into the medium. The medium containing the VLPs was clarified by centrifugation in a Beckman JA-14 rotor for 30 min at 12,000 rpm. The VLPs in the clarified supernatant were then pelleted through a 35% sucrose cushion by centrifugation in a Beckman SW28 rotor for 90 min at 25,000 rpm. The VLPs in the resulting pellets were suspended in CsCl in Tris-buffered saline, pH 7.4 (refractive index, 1.3610), and banded by isopycnic centrifugation in a Beckman SW50.1 rotor for 18 h at 35,000 rpm. The two visible bands seen for each preparation of double-layered VLPs were collected by side puncture and dialyzed extensively against 10 mM Tris-buffered saline, pH 7.4, and kept at 4°C until used.

**Production and purification of rotavirus single-layered VLPs.** Single-layered VLPs containing VP1/2/3, VP2/3, VP1/2, and VP2 were prepared by a combination of the method for production of VP2 particles (39) and the method outlined above. Sf9 cells were infected, virus was adsorbed, and the inoculum was removed by low-speed centrifugation as described above. The infected cell pellet was resuspended in 220 ml of TNM-FH–10% FBS insect medium and maintained in spinner flasks. The protease inhibitors aprotinin and leupeptin (0.5 μg/ml each) were added at 1 and 2 days postinfection, and the infected cells were harvested at 3 days postinfection.

The single-layered VLPs remained cell associated. The infected cells were collected by centrifugation in a Damon IEC PR-6000 centrifuge for 15 min at 1,500 rpm, washed twice with cold phosphate-buffered saline, and suspended in 30 ml of 1% DOC–TED lysis buffer (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 1% sodium deoxycholate, 0.5 μg of aprotinin per ml, 0.5 μg of leupeptin per ml) for 2 min to swell the cells. The cell suspensions were lysed by vortexing for 1 min, which was followed by sonication for 3 min. Alternatively, cells were lysed with 1% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; Sigma, St. Louis, Mo.) being used in place of sodium deoxycholate. VLPs were purified from these lysates by the method described for double-layered VLPs. A single visible band was observed for each single-layered VLP type. The band was collected by side puncture and dialyzed extensively against 10 mM Tris-buffered saline, pH 7.4. The VLPs were kept at 4°C until used.

**Production and purification of VP6 complexes.** Sf9 cells were infected with pAc461/SA11-6 for the expression of double-layered particles as described above. The infected cells were maintained in a spinner culture in 220 ml of optimized serum-free medium Sf-900 II SFM (GIBCO, Grand Island, N.Y.) and harvested 6 days postinfection.

VP6 was released into the medium. Since expressed VP6 molecules are capable of forming oligomers (14), a physical method for the purification of oligomeric VP6 was adopted. The medium containing VP6 was clarified by centrifugation in a Beckman JA-14 rotor for 30 min at 12,000 rpm. Oligomeric VP6 in the clarified supernatant was pelleted through a 35% sucrose cushion by centrifugation in a Beckman SW28 rotor for 90 min at 25,000 rpm. The VP6 oligomers in the resulting pellet were suspended in CsCl in Tris-buffered saline, pH 7.4 (refractive index, 1.3610), and banded by isopycnic centrifugation in a Beckman SW50.1 rotor for 18 h at 35,000 rpm. A wide diffuse band was observed and collected by side puncture, dialyzed extensively against 10 mM Tris-buffered saline, pH 7.4, and kept at 4°C until used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining showed that the protein was >95% pure and boiled VP6 sample had the electrophoretic mobility of monomeric VP6 whereas the unboiled sample had the mobility of trimeric VP6.

**SDS-PAGE.** The protein profiles of all VLPs and protein complexes were determined by reducing SDS-PAGE analysis with 12% resolving and 4% stacking polyacrylamide gels as previously described (4, 22). Gels were stained with a silver staining kit (Sigma) according to the manufacturer's instructions.

**GTP binding.** The presence of VP3 in VLPs or complexes was determined by a GTP binding assay. These reactions were performed in a 20-μl reaction volume containing ~3.5 μg of VLP protein, 2 μCi of [α-$^{32}$P]GTP (Amersham), 2 mM $MgCl_2$, and 10 mM Tris-HCl-buffered saline, pH 7.4, as described by Liu et al. (23). After a 30-min incubation at room temperature, the reactions were terminated by the addition of 5 μl of 5× Laemmli sample buffer. After being boiled for 2 min, the proteins were resolved by SDS–12% PAGE and electroblotted onto a nitrocellulose membrane (37). [α-$^{32}$P]GTP binding proteins were visualized by autoradiography.

**EM.** The morphology of all VLP preparations was examined by electron microscopy (EM) (39). Copper grids coated with collodion-carbon and freshly glow discharged were used for sample adsorption. Each grid was floated on a drop of sample for 30 min, excess fluid was removed by blotting with a filter paper, and the grid was washed for 2 s on a drop of water, floated on a drop of 1% ammonium molybdate for 15 s, and air dried. All electron micrographs were taken with a Philips CM10 electron microscope operating at 80 kV.

**Replicase activity of VLPs and complexes.** A template-dependent in vitro replication system (8) was used to test the replicase activity of the various VLPs and VP6 complexes. The in vitro system, which contained [α-$^{32}$P]UTP and 400 ng of double-layered VLPs or 800 ng of single-layered VLPs (determined by Bio-Rad protein assay), was programmed with a reporter mRNA transcript and incubated for 4 h at 37°C, and the reaction products were resolved by SDS-PAGE as described previously (8). The presence of dsRNA product in the gel was determined by autoradiography and comparison with $^{32}P_i$-labelled rotavirus strain OSU dsRNA markers. The reporter RNA tested was derived by in vitro transcription of a plasmid vector containing the complete OSU genome segment 9 (pUC/OSU9) (8) following restriction of the plasmid with *Sac*II. This template RNA, designated OSU9, contained bona fide rotavirus 5′ and 3′ termini and replicated in the in vitro system (8). A negative control reporter RNA resulted from the transcription of pUC/OSU9 after restriction with *Xba*I, which resulted in the production of transcripts lacking the 3′-terminal 26 nucleotides of OSU9 mRNA. This control template RNA, OSU9-3′Xba, was not detectably replicated in the in vitro system (8). All double-layered VLPs were tested in replicate experiments, as were the single-layered VLPs (with double-layered VLPs being used as controls). In each experiment, a master mix containing all components was divided into aliquots, and the reaction was initiated by addition of the VLPs.

## RESULTS

**Double-layered VLPs. (i) Purification and EM characterization of double-layered VLPs.** To produce double-layered VLPs, Sf9 cells were coinfected with various combinations of baculovirus recombinants which encode rotavirus VP1, VP2, VP3, and VP6. All double-layered VLPs were released into the medium and were purified by CsCl gradients and analyzed by EM and SDS-PAGE.

Coexpression of VP1, VP2, VP3, and VP6 resulted in the formation of VP1/2/3/6 particles, which separated into two bands in CsCl gradients. The densities were 1.274 g/cm$^3$ for the light particles and 1.298 g/cm$^3$ for the heavy particles. Both light particles (Fig. 1A) and heavy particles (Fig. 1B) had structural features similar in size and appearance to those of empty native double-layered rotavirus particles (Fig. 1C). Coexpression of VP2, VP3, and VP6 resulted in the formation of VP2/3/6 particles, coexpression of VP1, VP2, and VP6 resulted in the formation of VP1/2/6 particles, and coexpression of VP2 and VP6 resulted in the formation of VP2/6 particles. VLPs containing VP1/2/6, VP2/3/6, and VP2/6 each resolved into two bands in CsCl gradients. In each of these cases, the light particles had a density of 1.273 g/cm$^3$ and the heavy particles had a density of 1.286 g/cm$^3$. Both the light and heavy particles of each type had structural features similar in size and appearance to those of empty native double-layered particles (Fig. 1C), as was shown for light particles of VP2/3/6 (Fig. 1D), light particles of VP1/2/6 (Fig. 1E), and light particles of VP2/6 (Fig. 1F; heavy particles are not shown). EM analyses of purified VP1/2/3/6, VP2/3/6, VP1/2/6, and VP2/6 particles stored at 4°C for at least 1 year revealed that the particles remained intact and thus are morphologically very stable (data not shown). The yield of each of these double-layered VLPs was approximately

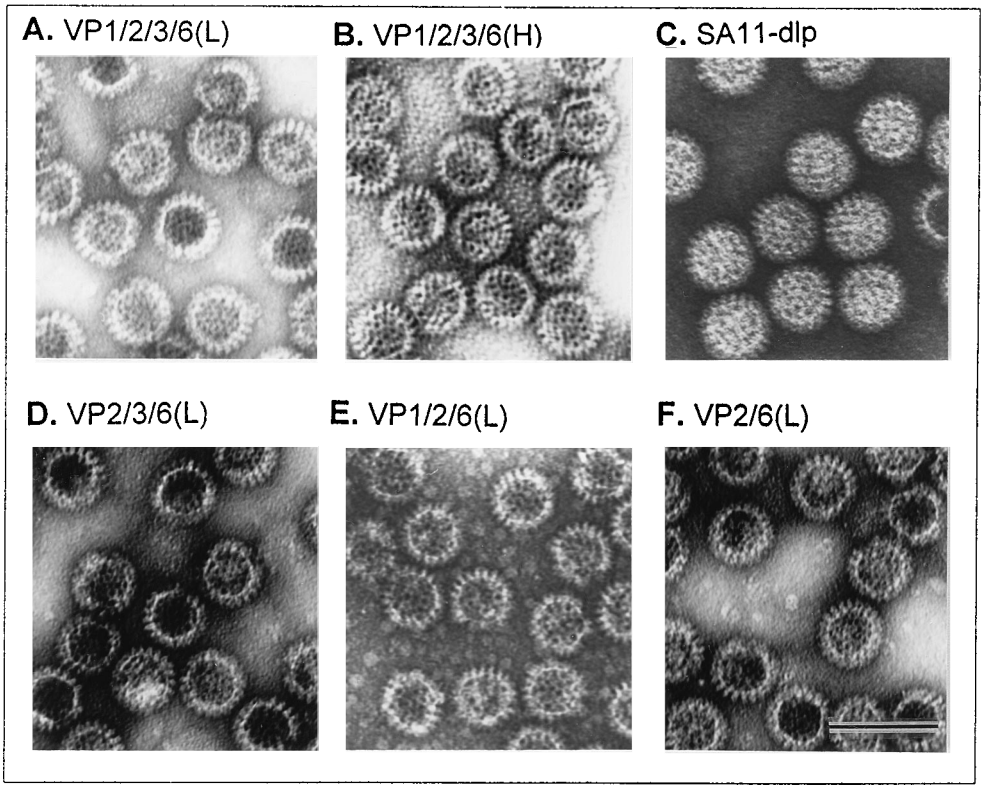


FIG. 1. Electron micrographs of the rotavirus double-layered VLPs and native particles. Shown are the negative-stain structures of the VLPs containing the indicated protein species. (L) and (H) refer to light and heavy particles in CsCl gradient purification. SA11-dlp, SA11 double-layered particles (formerly single-shelled particles). All micrographs are at the same magnification. Bar = 100 nm.

150 μg for the light band and 450 μg for the heavy band when $6 \times 10^8$ cells were infected.

**(ii) Protein composition of double-layered VLPs.** Purified, double-layered VP1/2/3/6, VP1/2/6, VP2/3/6, and VP2/6 particles were analyzed by SDS-PAGE and silver staining to confirm the presence of each structural protein in each VLP type (Fig. 2A). The presence of VP3 in VP1/2/3/6 and VP2/3/6 particles was confirmed by GTP binding (Fig. 2B, lanes 3 and 4), because it is present in minute amounts and has an electrophoretic mobility similar to that of VP2 degradation products (8, 39). Each of the proteins expected was structurally incorporated into the appropriate VLP type. There was no obvious difference in the protein profiles of the light and heavy VLPs of each type (Fig. 2A). The daily addition of protease inhibitors following infection of the insect cells resulted in VLPs with very little degradation of VP2 compared with that found in previous reports (8, 39).

The silver-stained gels were scanned and the amounts of VP1 and VP2 were normalized to the content of VP6 in each sample. The VP1:VP6 and VP2:VP6 ratios were relatively constant for all VLPs and were quite similar to those of native double-layered particles (Fig. 2A). Thus, the stoichiometry of proteins within the various double-layered VLPs closely approximates that found in native virus particles.

**(iii) Replicase activity of double-layered VLPs.** Purified, double-layered VP1/2/3/6, VP1/2/6, VP2/3/6, and VP2/6 particles and VP6 protein complexes were tested in the in vitro replication system for their ability to stimulate the incorporation of [α-$^{32}$P]UTP into dsRNA. Each particle type was tested with two template RNAs. OSU9 template RNA has bona fide 5′ and 3′ termini and is a competent replication substrate, whereas OSU9-3′Xba template RNA is truncated by 26 nucleotides at the 3′ terminus and cannot be replicated (8). As can be seen in Fig. 3 (lane 10), OSU9 template RNA was replicated by VP1/2/3/6 particles which were directly dialyzed against low-ionic-strength buffer and not purified further,

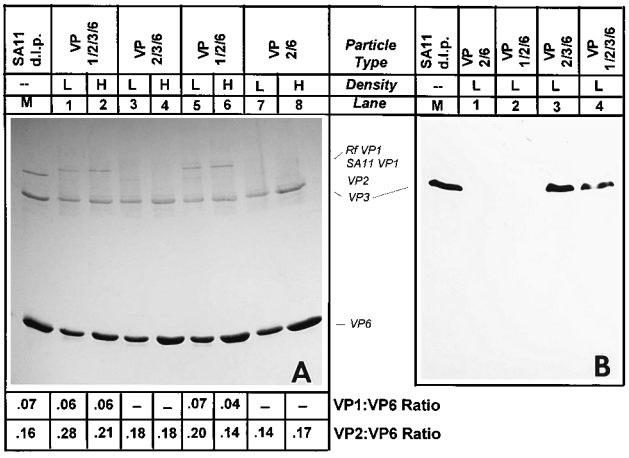


FIG. 2. Protein content of double-layered VLPs. (A) Silver-stained SDS–12% PAGE gel with the proteins of purified light and heavy VLPs resulting from the expression of the indicated genes. The ratios of VP1:VP6 and VP2:VP6 for each particle type as determined by scanning densitometry of the gel shown are given at the bottom. (B) Autoradiograph of a [$^{32}$P]GTP binding assay performed on the light-density VLPs resulting from the expression of the indicated genes. The positions of the visible rotavirus proteins are indicated between the panels. Lanes M contain native SA11 double-layered particles (d.l.p.) (formerly single-shelled particles) run as protein markers. (L) and (H) refer to light or heavy particles in CsCl gradient purification.

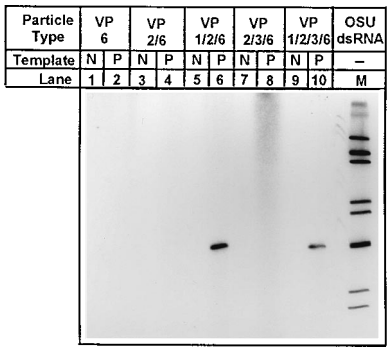


FIG. 3. Replicase activity of light-density double-layered VLPs. Shown are the replicase products of in vitro replication reactions in which the indicated double-layered VLP was used as the source of replicase activity. The products of the replicase reaction were labeled with [$\alpha$-$^{32}$P]UTP, resolved by SDS–10% PAGE, and subjected to autoradiography. Two different template RNAs were tested with each VLP type. N indicates a nonreplicating template (OSU9-3′Xba), and P indicates a replicating template (OSU9) as previously determined (8). Lane M contains $^{32}P_i$-labelled OSU dsRNA run as a marker.

rather than having VP6 removed by prior treatment with 1 M $CaCl_2$. These particle preparations contained a mixture of mostly intact double-layered particles with relatively few disrupted particles (data not shown). OSU9 was also a functional template in combination with VP1/2/6 particles (Fig. 3, lane 6), indicating that VP3 is not a necessary component of the particulate replicase. VP2/6 and VP2/3/6 particles were unable to stimulate the replication of OSU9 (Fig. 3, lanes 4 and 8), indicating that VP1 is a required constituent of the particulate replicase. VP6 complexes alone did not function as replicase when programmed with OSU9 template (Fig. 3, lane 2). As expected, none of the VLPs had replicase activity when programmed with OSU9-3′Xba templates (Fig. 3, lanes 1, 3, 5, 7, and 9). Since the reactions were programmed with equal amounts of VLPs (400 ng) and the VP1:VP6 ratio was constant in the VP1-containing VLPs (Fig. 2A), it appears that the particle-associated VP1 has replicase activity that is relatively constant per unit of VP1.

**Single-layered VLPs. (i) Purification and EM characterization of single-layered VLPs.** To produce single-layered VLPs, Sf9 cells were coinfected with various combinations of baculovirus recombinants which express VP1, VP2, and VP3. Single-layered VLPs remained associated with the infected cells and were released with hypotonic, detergent-containing lysis buffer prior to purification and analysis by EM and SDS-PAGE.

Coexpression of VP1, VP2, and VP3 resulted in the formation of VP1/2/3 particles, coexpression of VP2 and VP3 resulted in the formation of VP2/3 particles, and coexpression of VP1 and VP2 resulted in the formation of VP1/2 particles (Fig. 4). Following CsCl density gradient centrifugation, the VP1/2/3, VP2/3, and VP1/2 VLPs formed a single visible band with a density of 1.298 g/cm$^3$ in each case. EM revealed that the purified single-layered VLPs were in small aggregates (Fig. 4). The VP1/2/3 (Fig. 4A), VP2/3 (Fig. 4B), and VP1/2 (Fig. 4C) particles had structural features similar to those described for VP2 particles (Fig. 4D) (39) and native single-layered rotavirus particles (Fig. 4E) (8). The yield of each of these single-layered VLP types was approximately 200 $\mu$g from $6 \times 10^8$ infected cells. Release of VLPs in detergent-containing buffer allowed the purification of VLPs with a much lower level of aggrega-

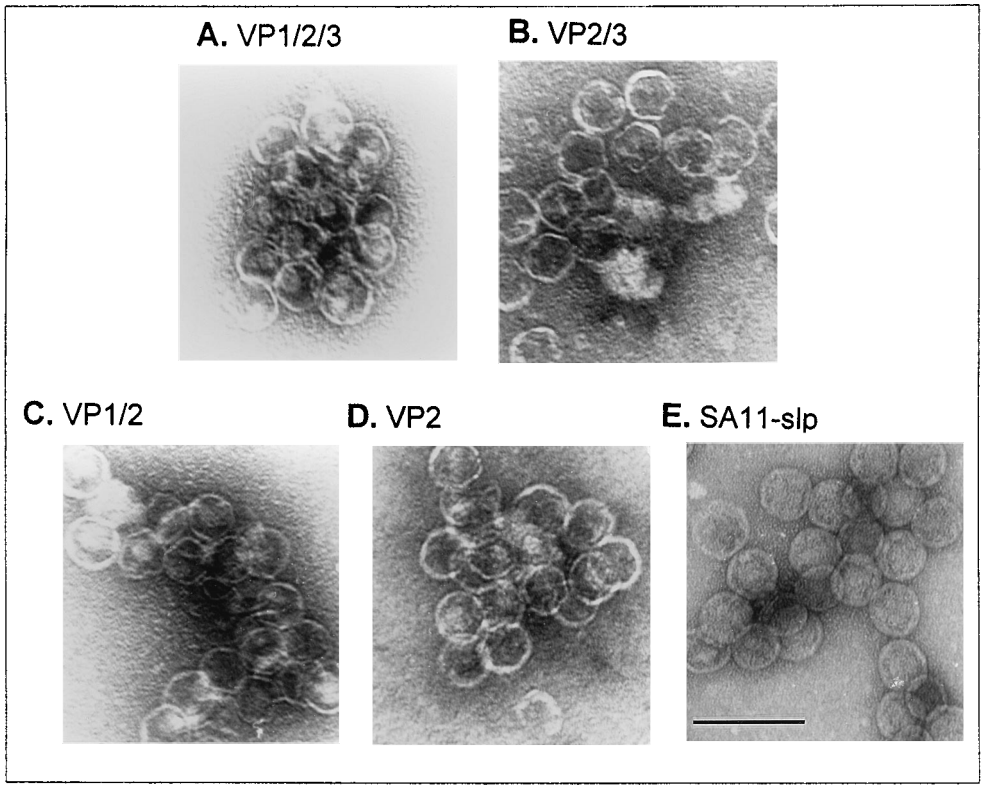


FIG. 4. Electron micrographs of rotavirus single-layered VLPs and native particles. Shown are the negative-stain structures of the VLPs containing the indicated protein species. SA11-slp, SA11 single-layered particles (cores). All micrographs are at the same magnification. Bar = 100 nm.

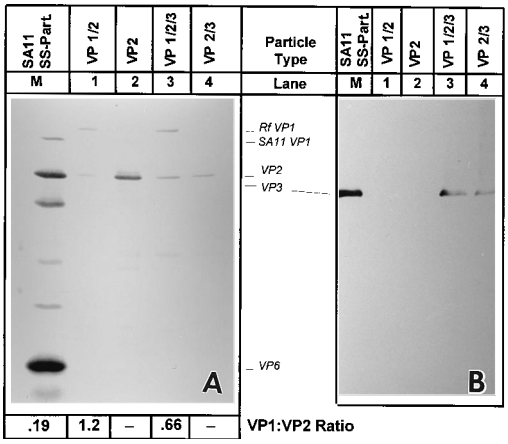


FIG. 5. Protein content of single-layered VLPs. (A) Silver-stained SDS–12% PAGE gel with the protein of purified VLPs resulting from the coexpression of the indicated genes. The ratios of VP1:VP2 for each particle type as determined by scanning densitometry of the gel shown are given at the bottom. (B) Autoradiograph of a [$^{32}$P]GTP binding assay performed on the VLPs resulting from the expression of the indicated genes. Lanes M contain SA11 double-layered particles (SS-Part.) run as protein markers. The positions of the relevant visible rotavirus proteins are indicated between the panels.

tion compared with that reported previously (8, 39). These VLP preparations did not precipitate $Cs^+$ ions during CsCl gradient centrifugation, indicating that very little free detergent remained following pelleting through a sucrose cushion. However, some detergent may remain complexed with the VLPs. Similar results were obtained whether 1% sodium deoxycholate or 1% CHAPS was the detergent.

**(ii) Protein composition of single-layered VLPs.** Purified, single-layered VP1/2/3, VP2/3, VP1/2, and VP2 (39) particles were analyzed by SDS-PAGE and silver staining to confirm the presence of each structural protein in each VLP type (Fig. 5A). The presence of VP3 in VP1/2/3 and VP2/3 particles was confirmed by GTP binding (Fig. 5B, lanes 3 and 4), because it is present in small amounts and has electrophoretic mobility similar to that of VP2 degradation products (8, 39). Each of the proteins expected was structurally incorporated into the appropriate VLP type (Fig. 5). Inclusion of protease inhibitors during growth and lysis of the infected insect cells resulted in VLPs with very little degradation of VP2 compared with that found in previous reports (8, 39). Scanning of silver-stained gels revealed significant variation in the VP1:VP2 ratios of the single-layered VP1-containing VLPs (Fig. 5A). The VP1-containing single-layered VLPs contained significantly more VP1 relative to VP2 than did control native triple-layered particles.

**(iii) Replicase activity of single-layered VLPs.** Purified single-layered VP1/2/3, VP2/3, VP1/2, and VP2 particles were tested in the in vitro replication system for the ability to stimulate the incorporation of [$\alpha$-$^{32}$P]UTP into dsRNA. Each particle type was tested with two template RNAs, OSU9 and OSU9-3′Xba, as described above. OSU9 was replicated when the in vitro reaction was programmed with VP1/2/3 particles (Fig. 6, lane 5), confirming prior results (8). In addition, OSU9 was replicated when VP1/2 particles were added to the system (Fig. 6, lane 1). However, OSU9 was not replicated when the system was programmed with VP2/3 or VP2 particles (Fig. 6, lanes 3 and 7). These results confirm the results obtained for double-layered VLPs and indicate that VP1 is a required component of the particulate replicase whereas VP3 is not. As expected, the OSU9-3′Xba template was not replicated to detectable levels by any of the VLP preparations tested (Fig. 6, lanes 2, 4, 6, and 8).

## DISCUSSION

Baculovirus recombinant vectors were developed as a tool which allows the introduction, maintenance, and high-level expression of foreign genes (36). A number of genes from more than a dozen human and animal viruses have been inserted into baculovirus vectors and expressed successfully in the insect cell expression system. In general, the expressed proteins maintain the immunogenicity and biochemical properties of their authentic viral counterpart proteins, and many capsid proteins assemble into VLPs. Several studies have shown that rotavirus structural proteins expressed from baculovirus vectors, singly or in coinfections, assemble into VLPs (10, 14, 21, 34, 39).

Here, we investigated the formation of VLPs when the five protein species of the inner two capsid layers of the rotavirus virion were coexpressed from baculovirus vectors. The goal was to determine the requirements for inclusion of minor core proteins VP1 and VP3 into VLPs. In addition, particles containing VP1, VP2, and VP3, derived from either native virions or from baculovirus coexpression, were recently shown to possess replicase activity (8). A second goal was to determine if any subset of the rotavirus structural proteins would have replicase activity or if VP1/2/3 particles constituted the minimal replicase.

Most of the rotavirus structural proteins, VP1, VP2, VP3, and VP6, assembled into VLPs when coexpressed from baculovirus vectors in combinations representing the components of the single-layered capsid (core) or the double-layered capsid (single-shelled particle). However, several points must be made relative to these particles. (i) Double-layered VLPs with VP6 on their surfaces were found in the medium of the infected cells, whereas single-layered VLPs, lacking VP6, remained cell associated. This suggests that VP6 is required on VLPs for release from cells. (ii) Neither VP1 nor VP3 could be incorporated into VLPs in the absence of VP2. Similarly, VP1/3 VLPs or complexes could not be isolated by our centrifugation techniques (data not shown). This suggests that VP2 acts as a scaffold onto which VP1 and VP3 are assembled.

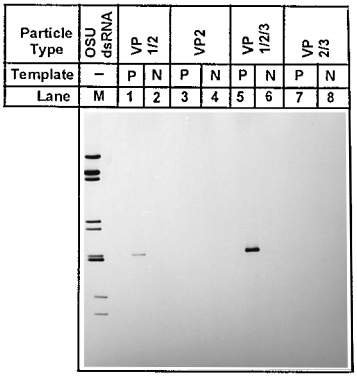


FIG. 6. Replicase activity of single-layered VLPs. Shown are the replicase products of in vitro replication reactions in which the indicated single-layered VLP was used as the source of replicase activity. The products of the replicase reaction were labeled with [$\alpha$-$^{32}$P]UTP, resolved by SDS–10% PAGE, and subjected to autoradiography. Two different template RNAs were tested with each VLP type. P indicates a replicating template (OSU9), and N indicates a nonreplicating template (OSU9-3′Xba) as previously determined (8). Lane M contains $^{32}P_i$-labelled OSU dsRNA run as a marker.

(iii) Double-layered VLPs could be separated into two distinct density fractions on CsCl gradients. Previously, we suggested that the density difference between the two populations was related to the relative amounts of VP2 degradation product present in each fraction (8). However, here we were able to isolate double-layered VLPs with significantly less VP2 degradation than previously, and the density difference remained. We have stored light and heavy double-layered VLPs for 1 year at 4°C and found that 90 to 95% of them are morphologically stable as estimated by negative-stain EM, although fewer than 50% of them will withstand lyophilization (38b). Thus, the basis for two populations of double-layered VLPs is unknown. The single density of single-layered VLPs suggests that VP6 plays a role in the two-density populations of double-layered VLPs. Support for this notion comes from studies of the density of reassortant double-layered particles in which the parental origin of VP6 played a partial role in the density differences observed among virion-derived double-layered reassortant particles (5). (iv) Single-layered VLPs were aggregated when purified, whereas double-layered VLPs were not. This suggests that single-layered VLPs have a hydrophobic surface structure. (v) In this study and others (10), rotavirus structural proteins efficiently assembled into VLPs when they were coexpressed from vectors containing single genes. This result contrasts with the reported requirement in the bluetongue virus system for the expression of multiple proteins from a single baculovirus vector for efficient assembly of VLPs (2, 16, 18, 28). In our experience, VLPs form efficiently, provided cells are infected with each recombinant at a high multiplicity of infection.

Examination of both single-layered and double-layered VLPs in the in vitro replication system revealed that only particles which contained both VP1 and VP2 had replicase activity, regardless of their content of other protein species. On this basis, we conclude that VP1/2 particles constitute the minimal replicase particle in the in vitro replication system. However, once again, these observations merit comment. (i) We previously found that open-core particles produced by stepwise disassembly of native virions contained only VP1, VP2, and VP3 in detectable amounts and had replicase activity in vitro. Sucrose gradient centrifugation and analysis of fractions by PAGE demonstrated that VP1, VP2, and VP3 were associated with the open-core particles that possessed replicase activity (8). We confirmed that other viral proteins present in trace amounts were not required for replicase activity by demonstrating that VP1/2/3/6 VLPs produced by baculovirus expression and treated in an identical manner also had replicase activity in vitro (8). The open-core particles produced by either of these methods were a mixture of disrupted and apparently intact but empty particles, as determined by EM (8). Here, we demonstrated that VP3 is not required in the VLPs for replicase activity and that particles containing only VP1 and VP2 had activity in vitro. To compensate for the small amount of VP3 present in the single- and double-layered VLPs, replicase assays were performed with double and triple concentrations of VP2/3 and VP2/3/6 particles. However, even under these conditions, no product was detected (data not shown). (ii) The description of the VP1/2 VLP as the minimal replicase particle contrasts with the work of Patton and Gallegos (17, 26, 27), who found that the earliest complex that could be isolated from infected cells and that was capable of replication in vitro contained the structural proteins VP1 and VP3 together with the nonstructural proteins NSP1, NSP2, NSP3, and NSP5. Pulse-chase studies showed that the sequential addition of VP2 and VP6, with the concomitant loss of nonstructural proteins, led to the formation of more mature particles with replicase activity in vitro. The difference between the definition of the minimal replication particle given in the cited studies and that given here is not clear and could be the subject of speculation. Here, we simply note that the particles examined by Patton and Gallegos were assembled in vivo and contained the template RNA upon which negative-strand synthesis had been initiated prior to isolation, whereas the particles we examined were expressed in vitro and both initiated and completed the synthesis of negative strands on exogenously added template RNA. (iii) VLPs containing VP1 and VP2 had greater replicase activity as double-layered VLPs (Fig. 3, lanes 6 and 10) than as single-layered VLPs (Fig. 6; compare lanes 1 and 5). Although the basis for this difference is not clear, we note several possible explanations. Single-layered VLPs required the presence of detergent for their initial release from cells, and this presence may have been detrimental to the recovery of enzymatic activity. The single-layered VLPs aggregated, and this aggregation may have made access of the template to the particles inefficient. In addition, the replicase reactions were programmed with twice the mass of particles as was used for double-layered VLPs and made less product. Finally, VP6 may play a role in maintaining the optimal conformation of the replicase, as it apparently does in the case of the transcriptase for which the addition of VP6 to single-layered (core) particles activates the transcriptase through what is thought to be a conformational effect (35). A combination of these factors may provide the explanation. (iv) The candidate protein for the rotavirus replicase is VP1, on the basis of the presence of conserved sequence motifs characteristic of RNA-dependent RNA polymerases (9). We were unable to isolate VLPs or protein complexes that contained only VP1 and made less product. VP3, or both (data not shown). Thus, we are currently unable to determine if VP1 alone is capable of replicating rotavirus template RNAs. It is possible that VP1 must be associated with VP2 in a structure to assume an active conformation, as cells expressing VP1 alone were unable to direct elongation in a primer-directed polymerization assay (9). Further work is needed to determine whether VP1 alone or in complexes with VP3 may have activity, as was suggested by the work of Patton and Gallegos. (v) We note that replicase-active particles were prepared from purified double-layered VLPs by direct dialysis against low-ionic-strength buffer, which produced replicase-active particles that contained VP6 as assayed by EM and PAGE. This method contrasts with our previous methods, in which VP6 was removed from double-layered VLPs by treatment with 1 M $CaCl_2$ prior to the low-ionic-strength dialysis step (8). As with our previous study (8), we dialyzed all VLPs against low-ionic-strength buffer prior to the analysis of replicase activity. Subsequently, we have found that double-layered VLPs have detectable replicase activity without dialysis. However, the replicase activity was very significantly increased if the VLPs were subjected to dialysis prior to the replicase assays. In contrast, no particle type derived from native virions possessed detectable replicase activity without prior dialysis (8, 38a). (vi) Finally, we note that open cores (VP1/2/3 particles) derived from native virions have significantly higher specific activity than do VLPs (VP1/2/3/6 VLPs) derived by baculovirus expression. In identical reactions programmed with 400 ng of replicase particle protein and 0.1 μg of OSU9 template RNA, open cores converted ~0.67% of template to dsRNA whereas VLPs converted only ~0.01% of template to dsRNA (38a).

In summary, we have expressed rotavirus proteins representing the species present in single- and double-layered capsids in the baculovirus system and purified and characterized the resulting VLPs. The majority of the combinations of coexpressed proteins assembled into VLPs in insect cells if the major struc-

tural protein of the inner layer (VP2) was present. The presence of VP2 was required for the incorporation of VP1 and VP3 into VLPs. The VLPs had replicase activity if both VP1 and VP2 were present in the structure. The presence of VP3 was unnecessary for the replicase activity of VLPs.

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