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Expression of Rotavirus VP2 Produces Empty Corelike Particles

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The complete VP2 gene of bovine rotavirus strain RF has been inserted into the baculovirus transfer vector pVL941 under the control of the polyhedrin promoter. Cotransfection of *Spodoptera frugiperda* 9 cells with wild-type baculovirus DNA and transfer vector DNA led to the formation of recombinant baculoviruses which contain bovine rotavirus gene 2. Infection of *S. frugiperda* cells with this recombinant virus resulted in the production of a protein similar in size and antigenic properties to the authentic rotavirus VP2. The protein binds double-stranded RNA and DNA in an overlay protein blot assay. Expressed VP2 assembles in the cytoplasm of infected cells in corelike particles 45 nm in diameter. These corelike particles were purified by sucrose gradient centrifugation and found to be devoid of nucleic acid. Coexpression of VP2 and VP6 from heterologous rotavirus strains (bovine and simian) resulted in the formation of single-shelled particles. These results definitively show the existence of an innermost protein shell in rotavirus which is formed independently of other rotavirus proteins. These results have implications for schemes of rotavirus morphogenesis.

Rotavirus, a member of the *Reoviridae* family, contains 11 segments of double-stranded RNA. The viral capsid consists of three layers of proteins (8). The most external layer, made up of VP4 and VP7, is easily removed in vitro by calcium chelation and very probably in vivo during the uncoating step of infection. The inner capsid is formed by VP6. Single-shelled particles possess transcriptase activity (5). Treatment of these particles with chaotropic agents removes the inner capsid and leads to core particles (1) which contain the proteins VP1, VP2, and VP3 and the 11 RNA segments. VP1 and VP3 are minor components of the central core (about 10 and 5%, respectively). These proteins may constitute the viral RNA polymerase complex, since they possess significant homology to other viral RNA-dependent RNA polymerases (4, 20, 22).

VP2 is the most abundant protein of the core (about 90%). Previous results have demonstrated that VP2 binds nucleic acids in a non-sequence-specific manner (2). Furthermore, nucleotide sequence analysis indicates that VP2 possesses two motifs present in various nucleic acid-binding proteins. (i) Two "leucine zippers" are present between amino acids 536 and 686 and could be implicated in dimerization of VP2 and enhancement of binding to nucleic acid (8). (ii) The region between amino acids 65 and 120 is predicted (14) to have an alpha-helix–turn–alpha-helix secondary structure, a motif found in several DNA-binding proteins. (iii) Finally, another sequence between amino acids 53 and 81 could also permit the dimerization of VP2. This sequence, predicted to be an alpha helix, contains five acidic amino acids and five lysines which are spaced exactly seven amino acids apart and hence could be viewed as being repeated every two turns in the alpha helix. This arrangement of oppositely charged amino acids might also allow dimerization of VP2 via alignment of parallel helices (22). These various motifs are conserved among the three rotavirus strains that have been sequenced so far (7, 17, 22). These observations suggest that VP2 may be the protein that binds to viral RNA; it could even be the protein necessary to induce additional bending of the double-stranded RNA segments, which cannot be packaged alone in the capsid because of their flexibility (16).

High-level expression of recombinant proteins in eucaryotic cells provides a good opportunity to investigate the functions and molecular interactions of proteins. The baculovirus expression system has been proved to be successful in expressing many proteins and particularly the proteins of the *Reoviridae*. Several of these recombinant proteins have been shown to be able to assemble in insect cells. For example, rotavirus VP6 and bluetongue virus (BTV) NS1 make tubules (9, 27), whereas BTV VP3 and VP7 form corelike particles when they are coexpressed (12).

To study VP2 and investigate the possibility that this protein forms structures, we expressed VP2 in insect cells. Recombinant baculoviruses containing the entire coding sequence of VP2 were constructed. Insect cells infected with these recombinants synthesize large amounts of protein which are assembled in corelike particles. Coinfection of insect cells with a recombinant expressing VP2 and another recombinant expressing VP6 (9) led to the formation of empty single-shelled particles.

MATERIALS AND METHODS

Virus and cells. The RF strain of bovine rotavirus was grown in cultures of MA104 cells as previously described (19). Single-shelled and double-shelled rotavirus particles were purified by centrifugation in cesium chloride gradients as previously described (5). Empty particles were found at the top of the cesium chloride gradient. Wild-type and recombinants of *Autographa californica* nuclear polyhedrosis virus (AcNPV) were grown in monolayers of *Spodoptera frugiperda* 9 cells as previously described (25). *S. frugiperda* 9 cells were grown and maintained in TNM-FH medium (25) containing 10% fetal bovine serum.

Synthesis and cloning of cDNA. A full-length clone corresponding to RNA segment 2 of the RF strain was obtained by synthetizing cDNA from the plus and minus strands with two oligonucleotidic primers corresponding to the 5′ (5′-CCG GGG CTA TTA AAG GT-3′) and 3′ (5′-CCG GGG TCA TAT CTC CAC AGT GGG GTT GGC-3′) end sequences of genomic segment 2. To each primer, the cohesive

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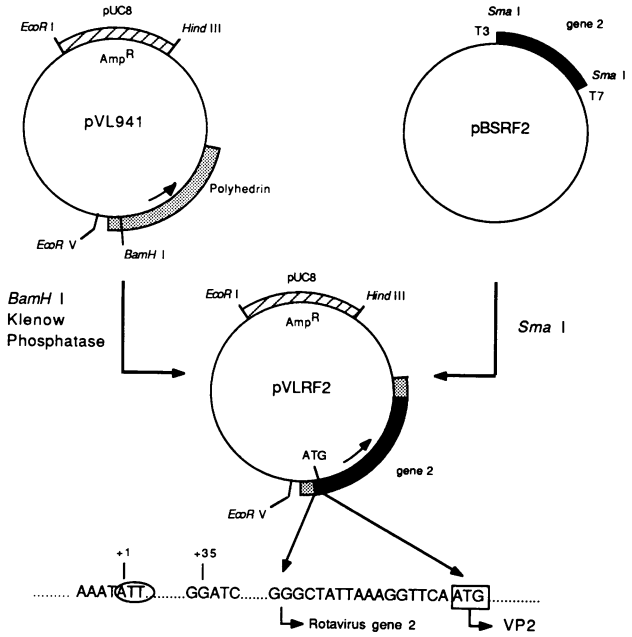

FIG. 1. Construction of transfer vector pVLRF2. Plasmid pBSRF2, containing the entire sequence of rotavirus gene 2, was constructed as described in Material and Methods. The *Sma*I insert of pBSRF2 was cloned into the Klenow-filled-in *Bam*HI site of transfer vector pVL941. Vector pVLRF2 contained gene 2 fused to the polyhedrin promoter. The mutated initiation codon of the polyhedrin gene is circled. Symbols: ▨, polyhedrin gene; ■, rotavirus gene 2.

sequence of the *Xma*I restriction site was added. The reannealed double-stranded cDNA was cloned in the unique *Xma*I site of pBS plasmid (Stratagene). One of the recombinant clones, pBSRF2, was partially sequenced to verify that the ends of the gene were complete.

Construction of baculovirus recombinants. The full-length clone pBSRF2 was digested with *Sma*I and subcloned into the Klenow-filled-in *Bam*HI site of transfer vector pVL941 (21; Fig. 1). After transformation into *Escherichia coli*, recombinant ampicillin-resistant colonies were screened for the correct orientation of the gene by restriction enzyme analysis.

Recombinant transfer vector and wild-type AcNPV DNA were used to cotransfect *S. frugiperda* 9 cells by the procedure described by Summers and Smith (25). Baculovirus recombinants were then screened by limiting dilution as previously described (4) and plaque purified three times by identifying polyhedrin-negative plaques. Stocks of three independent recombinant viruses designated BacRF2A, -B, and -C were prepared.

Protein analysis. *S. frugiperda* 9 cells infected with recombinant BacRF2A or with wild-type AcNPV at a high multiplicity (ca. 10 PFU per cell) were radiolabeled at various times postinfection (p.i.) with 20 μCi of [^{35}S]methionine (1,090 Ci/mmol) per ml of methionine-free Grace medium containing 10% fetal bovine serum. After 2 h, the cells were washed with unlabeled medium and harvested. Cellular proteins were solubilized in Laemmli buffer (18) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were either stained with Coomassie blue or impregnated with Amplify (Amersham) and exposed at −70°C to X-ray film.

In some experiments, cells were coinfected with BacRF2A and a recombinant (pAC461/SA11-6) which expresses the simian rotavirus VP6 (9). The multiplicity of infection was 10 PFU per cell for each recombinant, and cells were harvested 72 h p.i.

Production of antiserum. Rabbits shown to lack rotavirus antibodies were used to produce antiserum against recombinant VP2 protein. BacRF2A-infected *S. frugiperda* 9 cells (15×10^6 cells) were harvested 7 days p.i., pelleted, and suspended in phosphate-buffered saline containing 1% Nonidet P-40. Cells were sonicated, mixed with Freund's complete adjuvant, and inoculated intradermally. Two subsequent boosts with the same cell lysate in Freund's incomplete adjuvant were given every 3 weeks. Rabbits were bled 5 days after the last injection.

Western blot (immunoblot) analysis. Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membranes in a transfer buffer containing methanol (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with TS buffer (0.9% NaCl, 20 mM Tris-HCl [pH 7.4], 3% bovine serum albumin) for 30 min at 37°C, washed five times with T buffer (0.9% NaCl, 20 mM Tris-HCl [pH 7.4]), and incubated for 1 h at 37°C with rabbit anti-bovine RF serum (1/2,500) diluted in TS buffer or with anti-VP2 monoclonal antibodies (E22 kindly provided by R. L'Haridon [Jouy] or ABM4 kindly provided by H. Greenberg [Stanford]). After five washes in T buffer, alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G was added and incubated for 60 min at 37°C. Blots were washed again and developed with 4-Nitro Blue Tetrazolium chloride (220 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (255 mg/ml) in 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–50 mM $MgCl_2$ buffer.

Nucleic acid-protein-binding assay. Blots were prepared as described above and saturated for 30 min at 25°C in a standard binding buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.04% bovine serum albumin, 0.04% Ficoll, 0.04% polyvinylpyrrolidone). Radiolabeled DNA was synthesized by random priming purified rotavirus VP2 insert cDNA obtained from clone pBSRF2 as described by Feinberg and Vogelstein (11). Double-stranded RNA probes were prepared from purified rotavirus genomic RNA by radiolabeling with T4 RNA ligase and 5′-^{32}P-labeled cytidine biphosphate (3,000 Ci/mmol) as described by England and Uhlenbeck (6). Nitrocellulose membranes were incubated with probes diluted in standard binding buffer (10^5 cpm/ml) at 25°C for 1 h. Unbound probe was removed by three washes in standard binding buffer (2 min per wash) at 25°C. Blots were then dried and developed by autoradiography.

Solubilization and purification of VP2 particles. Cells infected by BacRF2A were harvested 3 days p.i. and suspended in 10 mM Tris-HCl (pH 7.4)–0.1 mM EDTA, and different solubilizing agents were added. Cell lysates were immediately clarified by centrifugation for 10 min at 10,000 × *g*, and the soluble material was analyzed by SDS-PAGE. Supernatants of cellular extracts (5×10^6 cells) solubilized in 1% sodium deoxycholate (DOC)–0.1% SDS in the presence of protease inhibitors (leupeptin [0.5 μg/ml] and antipain dihydrochloride [50 μg/ml]) were loaded on a 5 to 20% (wt/vol) sucrose gradient in TE buffer (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA) and centrifuged for 120 min in an SW40 rotor at 30,000 rpm. Fractions were collected, analyzed by SDS-PAGE, and examined by electron microscopy after being stained with 2% uranyl acetate or with 1% phosphotungstic acid (pH 7.5).

Analysis of VP2 particles by electrophoresis. Fractions

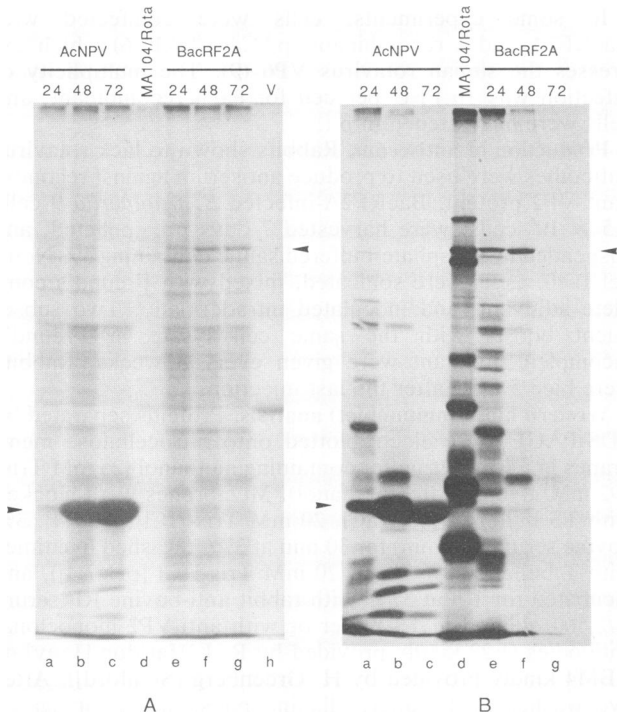

FIG. 2. Expression of VP2 in *S. frugiperda* cells. Proteins of cells infected by recombinant baculovirus BacRF2A or by baculovirus AcNPV were labeled at various times p.i. (24, 48, and 72 h) for 2 h with [^{35}S]methionine and resolved by electrophoresis on a 10% polyacrylamide gel. The gel was either stained with Coomassie blue (A) or developed by autoradiography (B). Polyhedrin and VP2 are indicated by arrowheads. Purified rotavirus RF strain (V) and MA104 cells infected with rotavirus were electrophoresed in lane h and lanes d, respectively.

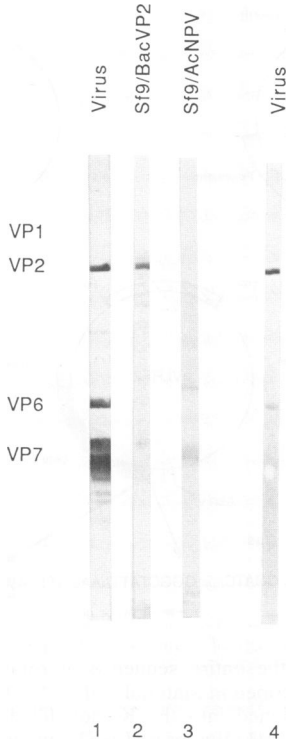

FIG. 3. Western blot analysis of VP2 protein. Proteins of rotavirus particles (lanes 1 and 4) or *S. frugiperda* 9 cells (Sf9) infected by BacRF2A (lane 2) or AcNPV (lane 3) were separated by SDS-PAGE and transferred onto nitrocellulose. Recombinant or native VP2 was detected by antirotavirus serum (lanes 1 to 3) or anti-recombinant VP2 serum (lane 4). In lane 2, several bands corresponding to the degradation of VP2 could also be detected.

containing VP2 particles or cesium chloride gradient-purified single-shelled and double-shelled particles were supplemented with 10% glycerol and loaded onto a Tris-glycine-agarose (TGA) gel (25 mM Tris, 192 mM glycine, 0.6% agarose). After electrophoresis, the gels were stained with Coomassie blue or ethidium bromide. Alternatively, proteins were transferred onto nitrocellulose membranes and immunostained as described above.

RESULTS

Expression of VP2 in *S. frugiperda* 9 cells. The coding sequence of a cDNA of rotavirus segment 2 was cloned into the transfer vector pVL941 (Fig. 1). Recombinants were obtained by cotransfection of *S. frugiperda* 9 cells with this vector and wild-type AcNPV DNA as described in Materials and Methods. To analyze the expression of VP2, infected cells were harvested at various times p.i., and the proteins were resolved by SDS-PAGE. As expected, in cells infected with recombinant virus, polyhedrin had disappeared and a new band with an apparent molecular weight of 94,000 was observed. This protein appeared as early as 24 h p.i. (Fig. 2A or B, lane e) and comigrated with the authentic protein found in virus particles (Fig. 2A, lane h) or with VP2 expressed in MA104 cells infected with the RF strain of bovine rotavirus (Fig. 2B, lane d). Three independent recombinant viruses (BacRF2A to BacRF2C) were initially identified and tested for their abilities to produce rotavirus VP2. One of these three recombinants expressed VP2 at a level 10-fold lower than those of the other two. One of the latter, BacRF2A, was characterized further.

Recombinant-infected *S. frugiperda* 9 cells, pulse labeled with [^{35}S]methionine, demonstrated that the rate of synthesis of VP2 was maximum at 48 h p.i. and very low at 72 h p.i. (Fig. 2B, lanes e to g). On Coomassie blue-stained gels (Fig. 2A, lanes e to g), it can be seen that VP2 accumulated in cells until 72 h p.i. Therefore, VP2 protein was shown to be stable in *S. frugiperda* 9 cells, since the amount of protein in stained gels did not decrease between 48 and 72 h p.i., whereas the rate of synthesis decreased after 48 h. Judged by visual comparison with purified protein standards, the amount of VP2 in cell extracts of BacRF2-infected *S. frugiperda* 9 was estimated to be 3 to 4 $\mu g/10^5$ cells. Extrapolation to a liter of suspension culture (2×10^9 cells) suggests that the level of VP2 expression was between 60 and 80 mg/liter of infected cells.

Antigenic and immunogenic characterizations of the recombinant protein. By Western blot analysis, we demonstrated that the recombinant protein reacted with a rabbit anti-bovine RF antiserum (Fig. 3, lanes 1 to 3) and a guinea pig anti-SA11 serum (data not shown). Monoclonal antibodies E22 and ABM4 against VP2 also reacted with the recombinant VP2 protein (data not shown). No protein of a similar molecular weight range present in insect cells infected with wild-type AcNPV reacted with the antirotavirus antibodies. Therefore, the protein of 94,000 is the authentic VP2 protein.

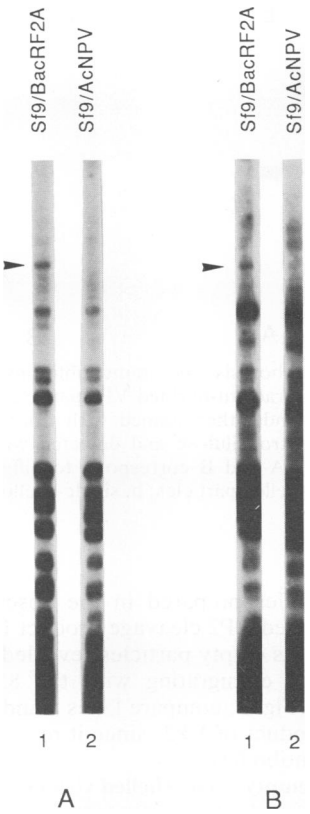

FIG. 4. Nucleic acid-binding properties of VP2 protein. Proteins of cells infected by BacRF2A (lanes 1) or AcNPV (lanes 2) were blotted onto nitrocellulose sheets. Blots were hybridized with rotavirus-specific double-stranded DNA (A) or rotavirus genome double-stranded RNA (B). VP2 is indicated by arrowheads.

Similarly, the recombinant protein was immunoprecipitated by an antirotavirus serum and detected by immunofluorescence staining in the cytoplasm of infected *S. frugiperda* 9 cells (data not shown).

An antiserum produced in rabbits against the recombinant VP2 also detected the viral protein after Western blotting (Fig. 3, lane 4).

Nucleic acid-binding properties of expressed VP2 protein. To verify that the recombinant protein was similar to the viral VP2, we investigated its ability to bind nucleic acid. Previously, it has been demonstrated by an RNA overlay protein blot assay that VP2 was able to bind nucleic acids in a nucleotide sequence-independent manner (2). Using the same conditions, we looked for the binding properties of the recombinant VP2 protein. *S. frugiperda* 9 cells infected with BacRF2A were harvested 48 h p.i., and the proteins were separated by SDS-PAGE and transferred onto nitrocellulose. Blots were incubated with various rotavirus-specific probes. The recombinant protein was able to bind double-stranded DNA, double-stranded RNA (Fig. 4), and single-stranded RNA (data not shown) probes.

Optimizing VP2 solubilization. Because the recombinant VP2 was not secreted into the culture medium and remained cell associated, even late after infection, the solubility of VP2 in the intracellular fraction was examined. Infected *S. frugiperda* 9 cells were harvested 40 and 72 h p.i., disrupted by sonication or freezing-thawing, and centrifuged for 10 min at 13,000 × *g*. The majority of VP2 was pelleted, probably in association with cellular components. Therefore, to solubilize the protein without dissociating (possible) multimers, VP2-expressing cells were treated with various detergents or lipid solvents before centrifugation. The pellet and supernatant fractions were analyzed by SDS-PAGE, and the solubility of VP2 was estimated after Coomassie blue staining (Table 1). Complete solubilization of VP2 in the monomeric form (data not shown) was achieved by treatment with ionic detergents (1% SDS). Among the mild detergents, only 1% DOC was able to solubilize a significant part of VP2. The addition of 0.1% SDS increased solubilization by DOC and did not disrupt particles. Surprisingly, the addition of two mild detergents (Triton X-100 and Brij 35) decreased the solubility of VP2.

TABLE 1. Solubilization of VP2

Solubilizing agent(s) (concn[a])	Solubility (%)[b]
None	0
n-Octyl D-glucopyranoside	0
Triton X-100	0
Brij 35	0
DOC	50
SDS	100
Taurine	0
CHAPSO	0
DOC + Triton X-100	0
DOC + Brij 35	0
DOC + 0.1% SDS	75

[a] For each solubilizing agent, the concentration was 1% unless specified. Brij 35, polyoxyethylene 23-lauryl ether; CHAPSO, 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate.

[b] Solubility was estimated as the percentage of VP2 remaining in the supernatant after centrifugation for 10 min at 10,000 × *g*.

When infected cells were freeze-thawed in the absence of protease inhibitors, we observed two additional products with apparent molecular weights of 90,000 and 85,000 (polypeptides I and II, respectively; data not shown). These new products reacted with the polyclonal antirotavirus serum or with anti-VP2 monoclonal antibodies and were, therefore, cleavage products of VP2. After solubilization of cells with 1% DOC, polypeptide I disappeared and the intensity of the polypeptide II band increased, suggesting that polypeptide I was an intermediate cleavage product. If infected cells were directly resuspended in Laemmli buffer, the recombinant protein had an apparent molecular weight of 94,000, which is similar to that of the viral protein (Fig. 2, compare lanes e, f, and g with lane h). Therefore, proteolytic cleavage occurred when the cells were disrupted. Various protease inhibitors were tested to prevent VP2 degradation (Table 2), and only cysteine protease inhibitors were efficient.

Purification and analysis of particles made from expressed VP2. To determine if VP2 was able to form structures in *S. frugiperda* 9 cells, soluble extracts of infected cells were loaded on 5 to 20% sucrose gradients. Each fraction was analyzed by SDS-PAGE to detect VP2.

VP2 was present as a broad peak in the middle of the sucrose gradient. Fractions containing VP2 were negatively stained and observed by electron microscopy. When uranyl acetate was used as a stain, these fractions appeared to contain large quantity of particles with diameters of about 45 nm (Fig. 5) and a hexagonal outlines similar to that observed with rotavirus core particles (1, 24). Corelike particles have an S value of 120 ± 10 as estimated by migration in a linear sucrose gradient. Such particles were not found when wild-

TABLE 2. Degradation of VP2[a]

Specificity of inhibitor	Protease inhibitor	VP2 cleavage
Serine protease	APMSF	+
	Aprotinin	+
Serine/cysteine protease	Antipain dihydrochloride	−
	Leupeptin	−
Cysteine protease	E64	−
Aspartate protease	Pepstatin	+
Metalloprotease	Bestatin	+
	EDTA	+

[a] To prevent VP2 cleavage during solubilization, various protease inhibitors (Boehringer Mannheim) were tested. Protease inhibitors were added to the cell lysates at the concentrations recommended by the manufacturer, and their effects were analyzed. Protease inhibitors are efficient when VP2 products I and II are not detected in SDS-PAGE. APMSF, (4-amidinophenyl)-methanesulfonylfluoride.

type baculovirus-infected *S. frugiperda* 9 cell lysates were used as controls. The presence of stain inside particles indicated that they did not contain nucleic acid. Similar structures were detected with phosphotungstic acid staining but only when the samples were fixed with 1% glutaraldehyde before being stained. Similar degradation with phosphotungstic acid has been described elsewhere for non-group A rotavirus (23, 26).

Particles observed by electron microscopy were also analyzed by electrophoresis in a TGA gel, which has been used to separate rotavirus subviral particles (13). A single band was observed when sucrose fractions containing VP2 were loaded onto TGA gels (Fig. 6A). This band reacted with antirotavirus serum after Western blotting (Fig. 6B); therefore, these particles are composed of the recombinant protein. Staining of the TGA gel with ethidium bromide failed to detect nucleic acid in these particles.

SDS-PAGE analysis of the sucrose gradient fractions showed that the top fractions did not contain VP2. The absence of monomeric forms in the cell lysate suggests a very efficient assembly of VP2.

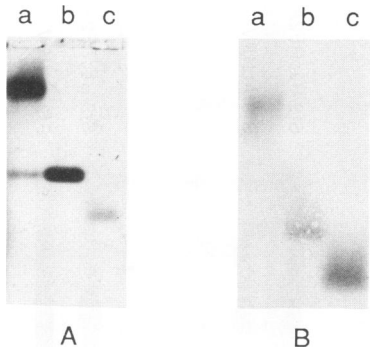

FIG. 6. Electrophoresis and immunoblotting of VP2 corelike particles. Sucrose gradient-purified VP2 particles were resolved on a 0.6% TGA gel and either stained with Coomassie blue (A) or transferred onto nitrocellulose and detected with anti-bovine RF serum (B). Panels A and B correspond to different experiments. Lanes: a, double-shelled particles; b, single-shelled particles; c, VP2 corelike particles.

Corelike particles prepared in the absence of protease inhibitors contained VP2 cleavage product II (85,000). SDS-PAGE of rotavirus empty particles revealed the presence of an extra protein comigrating with the 85,000-molecular-weight product (Fig. 7, compare lanes a and c). This protein is a cleavage product of VP2, since it reacts with monospecific anti-VP2 antibodies.

Formation of empty single-shelled virions by coexpression of VP2 and VP6 from heterologous rotavirus strains. Coinfection of *S. frugiperda* 9 cells with BacRF2A and pAC461/SA11-6 (which expresses VP6) resulted in the formation of particles with structures similar to those of single-shelled virions. These particles could be concentrated and purified by centrifugation on sucrose gradients (Fig. 8). Such particles formed readily, even though the expressed proteins were from heterologous (bovine and simian) rotavirus strains. Analysis of purified particles on Western blots using

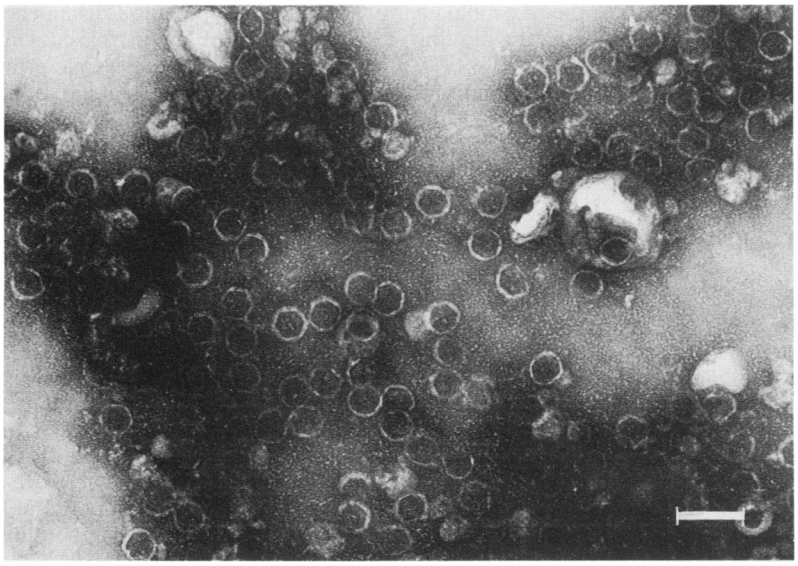

FIG. 5. Electron micrograph of purified VP2 particles. Lysate of BacRF2A-infected *S. frugiperda* 9 was analyzed on a sucrose gradient. Fractions containing VP2 were stained with 2% uranyl acetate and immediately examined. Bar = 100 nm.

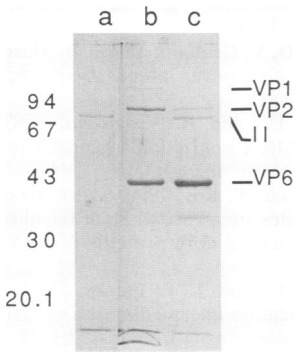

FIG. 7. Cleavage of VP2. Corelike particles purified in the absence of protease inhibitors (lane a), purified rotavirus single-shelled particles (lane b), and rotavirus empty particles (lane c) were analyzed by SDS-PAGE. Positions of molecular weight markers and of viral proteins are indicated. II, cleaved form of VP2.

polyclonal anti-SA11 serum showed the presence of VP2 and VP6 (data not shown).

DISCUSSION

The rotavirus VP2 protein has been expressed in the baculovirus expression system. A large amount of VP2 is synthesized. The recombinant protein is stable in infected *S. frugiperda* 9 cells and is similar in size to the authentic viral protein. The recombinant baculovirus-expressed VP2 protein also possesses a serological specificity similar to that of the viral VP2, since the recombinant protein is able to react with monoclonal and polyclonal antibodies raised against rotavirus VP2. In addition, antibodies raised against the baculovirus-expressed protein react with VP2 in rotavirus particles and infected mammalian cells. The recombinant protein, like the viral protein, is able to bind double-stranded DNA or RNA in an RNA overlay protein blot assay.

Particles very similar to rotavirus cores can be purified from recombinant baculovirus-infected cells by sucrose gradient centrifugation. These particles are recognized by an antirotavirus polyclonal antibody and anti-VP2 monoclonal antibodies in Western blot. Their diameter (45 nm) as determined by electron microscopy is very close to the diameter of rotavirus core particles prepared from single-shelled particles by treatment with chaotropic agents (1).

In TGA gels, rotavirus particles migrate according to their diameters (13), but VP2 particles migrate more slowly than expected and have a calculated diameter of 60 nm when double-shelled and single-shelled rotavirus particles are used as standards. This observation differs significantly from the electron microscopy determination, and the difference may be due to the absence of the 11 RNA segments and of the minor core proteins VP1 and VP3.

In the absence of protease inhibitors, corelike particles composed of polypeptide II could be purified, suggesting that VP2 may be cleaved without disruption of particles. Cleavage of VP2 has been reported previously (10), but its function remains unclear. Interestingly, VP2 found in empty particles is also cleaved, and two polypeptides of 90,000 and 85,000 are observed (3). Therefore, the cleavage of VP2 in the two kinds of empty particles suggests that the absence of nucleic acids interacting with VP2 makes the protein susceptible to proteases.

The assembly of VP2 expressed in insect cells into corelike particles demonstrates that this protein alone provides the structural integrity of core particles. Consequently, nonstructural proteins, minor core proteins, and nucleic acids are not required for the assembly of these particles. This observation is consistent with results obtain by electron cryomicroscopy (23a, 27), which suggest that VP2 makes a third shell between the inner shell composed of VP6 and the "subcore" composed of VP1, VP3, and the genome. The fact that VP2 makes particles alone contrasts with previous results obtained with BTV. For this virus, coexpression of VP3 and VP7 was reportedly needed to obtain corelike

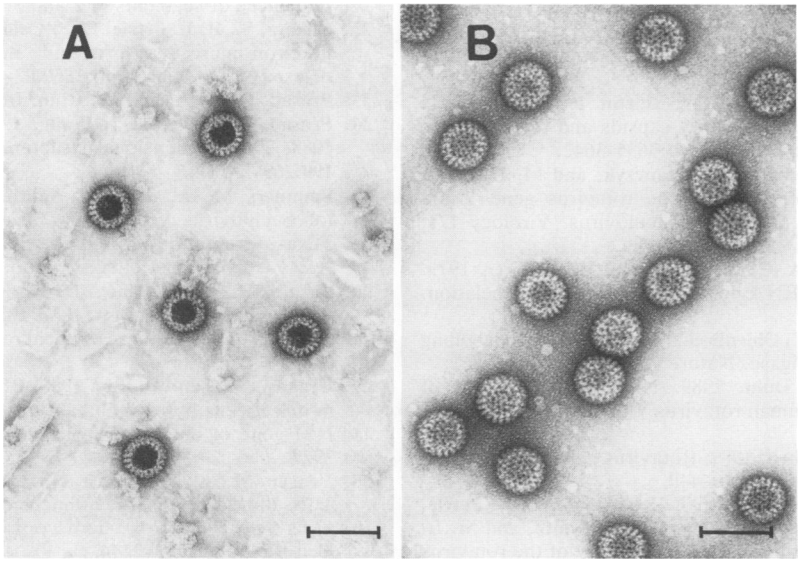

FIG. 8. Empty single-shelled particles formed by coexpression of heterologous VP2 and VP6. *S. frugiperda* 9 cells were coinfected with recombinant baculoviruses BacRF2A and pAC461/SA11-6. (A) These particles were purified on sucrose gradients and visualized by electron microscopy after being stained with 1% uranyl acetate. (B) Single-shelled particles purified from SA11-infected MA104 cells are shown for comparison. Bar = 100 nm.

particles (12). However, the corelike particles described for BTV are probably equivalent to rotavirus single-shelled particles, which are similarly produced by coexpression of rotavirus VP2 and VP6. The ability to produce single-shelled particles indicates that VP2 corelike particles are also capable of authentic protein-protein interactions, and formation of such particles does not require interactions with other viral components.

The absence of the monomeric form of VP2 in infected *S. frugiperda* 9 cells indicates that VP2 is rapidly and efficiently assembled into particles. The assembly of rotavirus core particles in infected mammalian cells represents a complex series of steps (13). The first subviral particles, precore RI, are composed of VP1, VP3, nonstructural proteins, and the positive RNA strands. The second step of the virus assembly consists in part in the addition of VP2 to the precore RI. Autoassembly of VP2 in insect cells in the absence of other viral components could indicate that in rotavirus-infected cells the precore RI binds to a polymeric form of VP2. Alternatively, one could hypothesize that nonstructural proteins and minor core proteins play a role in synchronizing the formation of particles by preventing autoassembly of VP2 before the formation of precore RI.

The high level of expression of VP2 by the recombinant baculovirus provides sufficient material for functional analysis and especially for studying the interactions of VP2 with genomic RNA and with other rotavirus core proteins which have also been expressed in the baculovirus system. It will be of interest to determine if the VP2 particles function as precursors into which viral RNAs are encapsidated, as has recently been shown to be the case for ϕ6 procapsids (15).

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REFERENCES

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. **58**:561–568.
3. **Brüssow, H., A. Bruttin, and S. Marc-Martin.** 1990. Polypeptide composition of the rotavirus empty capsids and their possible use as a subunit vaccine. J. Virol. **64**:3635–3642.
4. **Cohen, J., A. Charpilienne, S. Chimonczyk, and M. K. Estes.** 1989. Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology **171**: 131–140.
5. **Cohen, J., J. Laporte, A. Charpilienne, and R. Scherrer.** 1979. Activation of rotavirus RNA polymerase by calcium chelation. Arch. Virol. **60**:177–186.
6. **England, T. E., and O. C. Uhlenbeck.** 1978. 3′-Terminal labelling of RNA with T4 RNA ligase. Nature (London) **275**:560–561.
7. **Ernst, H., and J. A. Duhl.** 1989. Nucleotide sequence of genomic segment 2 of human rotavirus Wa. Nucleic Acids Res. **17**:4382.
8. **Estes, M. K., and J. Cohen.** 1989. Rotavirus gene structure and function. Microbiol. Rev. **53**:410–449.
9. **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.
10. **Estes, M. K., D. Y. Graham, and B. B. Mason.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. **39**:879–888.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **French, T. J., and P. Roy.** 1990. Synthesis of bluetongue (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. J. Virol. **64**:1530–1536.
13. **Gallegos, C. O., and J. T. Patton.** 1989. Characterization of rotavirus replication intermediates: a model for the assembly of single-shelled particles. Virology **172**:1–12.
14. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
15. **Gottlieb, P., J. Strassman, X. Qiao, A. Frucht, and L. Mindich.** 1990. In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage ϕ6: studies with procapsids assembled from plasmid-encoded proteins. J. Bacteriol. **172**:5774–5782.
16. **Kapahnke, R., W. Rappold, U. Desselberger, and D. Riesner.** 1986. The stiffness of dsRNA: hydrodynamic studies on fluorescence-labelled RNA segments of bovine rotavirus. Nucleic Acids Res. **14**:3215–3228.
17. **Kumar, A., A. Charpilienne, and J. Cohen.** 1989. Nucleotide sequence of the gene encoding for the RNA binding protein (VP2) of RF bovine rotavirus. Nucleic Acids Res. **17**:2126.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227**:680–685.
19. **L'Haridon, R., and R. Scherrer.** 1976. In vitro culture of a rotavirus associated with neonatal calf scours. Ann. Rech. Vet. **7**:373–381.
20. **Liu, M., and M. K. Estes.** 1989. Nucleotide sequence of the simian rotavirus SA11 genome segment 3. Nucleic Acids Res. **17**:7991.
21. **Luckow, V. A., and M. D. Summers.** 1988. High level expression of non-fused foreign genes with Autographa californica nuclear polyhedrosis virus expression vectors. Virology **170**:31–39.
22. **Mitchell, D. B., and G. W. Both.** 1990. Completion of the genomic sequence of the simian rotavirus SA11: nucleotide sequences of segments 1, 2 and 3. Virology **177**:324–331.
23. **Nakata, S., B. L. Petrie, E. P. Calomeni, and M. K. Estes.** 1987. Electron microscopy procedure influences detection of rotaviruses. J. Clin. Microbiol. **25**:1902–1906.

23a. **Prasad, B. V. V., and W. Chiu.** In press.

24. **Prasad, B. V. V., G. J. Wang, J. M. P. Clerx, and W. Chiu.** 1988. Three-dimensional structure of rotavirus. J. Mol. Biol. **199**:269–275.
25. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station bulletin no. 1555. Texas A&M University, College Station.
26. **Suzuki, H., G.-M. Chen, T. Hung, G. M. Beards, D. W. G. Brown, and T. H. Flewett.** 1987. Effects of two negative staining methods on the Chinese atypical rotavirus. Arch. Virol. **94**:305–308.
27. **Urakawa, T., and P. Roy.** 1988. Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10. J. Virol. **62**:3919–3927.
28. **Yeager, M., K. A. Dryden, N. H. Olson, H. B. Greenberg, and T. S. Baker.** 1990. Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. J. Cell Biol. **110**:2133–2144.