JOURNAL OF VIROLOGY, Sept. 1991, p. 4720–4727
0022-538X/91/094720-08$02.00/0

Vol. 65, No. 9

Rotavirus Protein Rearrangements in Purified Membrane-Enveloped Intermediate Particles

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Received 14 January 1991/Accepted 28 May 1991

Rotavirus, a double-shelled nonenveloped member of the *Reoviridae* family, becomes transiently membrane enveloped during its maturation process, as single-shelled particles bud from cytoplasmic viroplasm structures into the adjacent endoplasmic reticulum. The present study describes the isolation of these membrane-enveloped viral intermediates from rotavirus SA11-infected Ma104 cells. The enveloped intermediates comprised the proteins VP1, VP2, VP4, VP6, VP7, and NS28 and small amounts of NS35 and NS34. VP7 in the intermediate particles was recognized by either a polyclonal antibody to VP7, which previous studies had shown recognizes the membrane-associated form of VP7, or a monoclonal antibody which recognizes VP7 on mature virus. NS28, VP7, and VP1 to VP4 could be complexed to a higher-molecular-weight form when the membrane-permeable cross-linker dithiobis(succinimidylproprionate) was used. However, when an impermeable cross-linker was used, the structural proteins, including VP7, were not accessible to cross-linking. Velocity sedimentation of cross-linked immunoisolated enveloped virus particles showed that VP7 and VP4 were located in the same fractions only when the membrane-permeable cross-linker was used, implying their heterooligomeric association during outer capsid formation. When intermediate enveloped virus particles were treated with protease, VP6 and VP7 were protected, but not in the presence of detergent. Taken together, these results support the idea that in the membrane-enveloped intermediate, VP7 is repositioned from its location in the endoplasmic reticulum lumen back across the viral membrane envelope to the interior of the virus particle during the maturation process.

Rotavirus undergoes a unique mode of morphogenesis which is limited to specialized regions of the cytoplasm and to the endoplasmic reticulum (ER) (1, 7, 9, 13, 21). Inner capsid particles, made up of nucleic acid and the viral structural proteins VP1, VP2, and VP6, form in the amorphous viroplasm structures in the cytoplasm (13, 14). These single-shelled particles bud from the periphery of the viroplasm into the adjacent ER, mediated by the binding of VP6 to the nonstructural transmembrane glycoprotein NS28 (2, 3, 16). The virus thus becomes transiently enveloped by a portion of membrane derived from the ER and acquires viral proteins associated with this membrane. During the normal maturation process within the lumen of the ER, elements of the transient membrane envelope are lost and the viral proteins are rearranged so that VP7 (6) and VP4, the hemagglutinin (12), form the outer capsid. It has been claimed that the initial budding of rotavirus into the ER is a calcium-dependent process. The treatment of infected cells with tunicamycin (18, 20) or their incubation in calcium-free medium containing the ionophore A23187 (19) results in the arrest of rotavirus maturation in the membrane-enveloped intermediate form following its budding into the ER. The mechanisms underlying the loss of specific membrane elements, including lipids and proteins, and the retention of other viral proteins during the uncoating process are unknown. A logical approach to attempt to delineate the factors necessary for the rearrangement of membrane elements is to isolate this transiently membrane-enveloped intermediate to determine its composition, protein topologies, and in vitro uncoating requirements. In the present study, we devised a fractionation and immunoisolation procedure using disrupted fractions from rotavirus SA11-infected Ma104 cells, a polyclonal antibody to the virus nonstructural glycoprotein NS28, and an immunomagnetic matrix. The proteins of the immunoisolated virus particles were then assessed by immunoprecipitation, use of thiol-cleavable cross-linking agents, velocity sedimentation of the cross-linked complexed material, and analysis of protection to protease digestion.

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MATERIALS AND METHODS

Virus, radiolabeling of cells, and ionophore treatment. Ma104 cells were grown as monolayers in 100-mm dishes (Corning Glass Works, Corning, N.Y.) at 37°C in Dulbecco's modified Eagle medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 5% fetal calf serum, and 5% calf serum (all from GIBCO Laboratories, Grand Island, N.Y.). Simian rotavirus SA11 was propagated at a low multiplicity of infection in Ma104 cells as described previously (22). Cells were infected at a multiplicity of infection of 30 PFU per cell and were radiolabeled with Tran ^{35}S label ([^{35}S]-methionine and [^{35}S]-cysteine) (ICN, Cleveland, Ohio) for 2 h at 3 h after infection. Aliquots of infected-cell lysates radiolabeled in this fashion were utilized as markers for viral proteins. When utilized, ionophore A23187 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was applied 2 h after infection from a stock solution in dimethyl sulfoxide to a final concentration of 5 μM in calcium-free medium, for 3 h before fractionation of cellular membranes.

Fractionation and negative staining. At the end of the radiolabeling period, 15 dishes of cells were rinsed with

phosphate-buffered saline (PBS). Cells were harvested with a rubber policeman into PBS, pelleted at 2,000 rpm using an IEC, rotor type 269 for 5 min, and resuspended in several milliliters of 20 mM Tris-HCl (pH 8.2)–150 mM NaCl–1 mM $MgCl_2$ (MNT) buffer. The cells were then disrupted batchwise with a ball-bearing homogenizer (4) with eight strokes on the syringe plungers. A postnuclear supernatant was obtained after centrifugation at 2,000 rpm as above for 10 min and then was centrifuged at 9,000 rpm (9,770 × *g*) for 20 min at 4°C. The resulting pellet was resuspended in 1 ml of MNT buffer and sheared 10 times with a 26-gauge needle. This volume was then applied to the top of a sucrose step gradient (33% [wt/vol], 0.5 ml; 39%, 0.5 ml; 44%, 0.6 ml; 48%, 0.7 ml; 58%, 0.6 ml; and 68%, 0.6 ml) in MNT buffer and centrifuged in an SW60 rotor at 55,000 rpm (320,000 × *g*) for 2 h at 4°C. All bands appearing at the sucrose interfaces were removed with a sterile cannula from the top of the gradient. The volumes corresponding to the opaque bands removed from the 44%/48% and 39%/44% interfaces each had an equal volume of buffer added, were resheared with a 26-gauge needle, and were reapplied to the top of a second equivalent sucrose step gradient and centrifuged at 55,000 rpm (320,000 × *g*) for 2 to 3 h at 4°C. Two- to three-drop fractions were collected from the bottom of the tube by puncturing it with a 19-gauge needle. Material at the 44%/48% and 39%/44% interfaces, designated band 3 and band 4, respectively, had densities ranging from 1.18 to 1.21 g/ml, were pooled, and either were used immediately for immunoisolation or were stored at 4°C. The general organelle and viral content of this material was assessed by electron microscopy after negative staining. Aliquots of the gradients were diluted with 20 mM Tris-HCl–1 mM $MgCl_2$ (pH 8.2) buffer and pelleted in the airfuge at 20 lb/in^2 for 15 min and washed once. The pellets were resuspended in 20 μl of the same buffer, and the drop was applied to a carbon-coated copper grid and then negative stained with 2% phosphotungstic acid for 5 min. Grids were examined in a JEOL 1200 electron microscope to determine which fractions contained the greatest number of apparently intact membrane-enveloped intermediate virus particles.

Affinity purification of polyclonal anti-NS28 antibody. Proteins from unlabeled SA11-infected Ma104 cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 11% gel run alongside radiolabeled virus markers. The gel was incubated in several changes of 25 mM sodium phosphate (pH 6.5), and the proteins were then electrophoretically transferred to aminophenylthioether cellulose paper prepared by published methods (8, 17) and the manufacturer's recommendations (Schleicher & Schuell, Inc., Keene, N.H.). The paper was blocked in 10 mM Tris-HCl (pH 8.8)–1% bovine serum albumin (BSA)–10% ethanolamine for 3 h at 4°C and then rinsed in 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–1% BSA (TBS). It was then dried and exposed to Kodak SB5 film, and the band corresponding to NS28 was cut out of the paper and incubated with the polyclonal antiserum, made against the entire NS28 protein fused to β-galactosidase (kindly provided by J. Rothblatt, Department of Biochemistry, University of California, Berkeley), in TBS overnight at 4°C. Following several washes in TBS containing 0.05% Tween 20 or in TBS alone, the paper was treated with 4 M guanidine hydrochloride in PBS to remove the specifically bound antibody from the paper. The volume containing the affinity-purified anti-NS28 antibody was dialyzed against PBS and stored at −70°C.

Immunoisolation of membrane-enveloped virus. The fractions containing enveloped virus particles were diluted twofold with PBS containing 0.5% BSA (PBS-BSA) (10) and resheared with a 26-gauge needle. An aliquot of preimmune antiserum from a rotavirus-free rabbit, polyclonal antiserum to NS28, or affinity-purified antibody to NS28 was added, and the mixtures were incubated on a rotator at 4°C for several hours or overnight. Immunomagnetic Dynal M450 particles (Dynal Inc., Oslo, Norway), 4.5 μm in diameter, coated with 5 μg of sheep anti-rabbit immunoglobulin G (IgG) per mg of beads were washed several times in PBS-BSA and then added to the fractions containing the anti-NS28 antibody; they were then incubated on an end-over-end rotator at 4°C for several hours. The Dynal particles containing the immunoisolated material were pulled to the side of each Eppendorf tube with a hand-held magnet, and the supernatant was removed. The magnetic matrix was resuspended in PBS-BSA and incubated on a rotator for 30- to 60-min intervals at 4°C for several such washes, the last of which was performed with PBS. The Dynal matrix with specifically bound material was aliquoted. One aliquot was fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, treated with osmium tetroxide, embedded in Epon, and thin sectioned for examination by electron microscopy. Another aliquot of specifically bound material was solublized with 1% Triton X-100 (TX-100) in PBS and analyzed by SDS-PAGE as described above on 10 or 11% SDS-polyacrylamide gels.

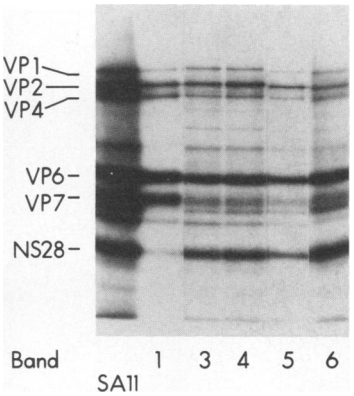
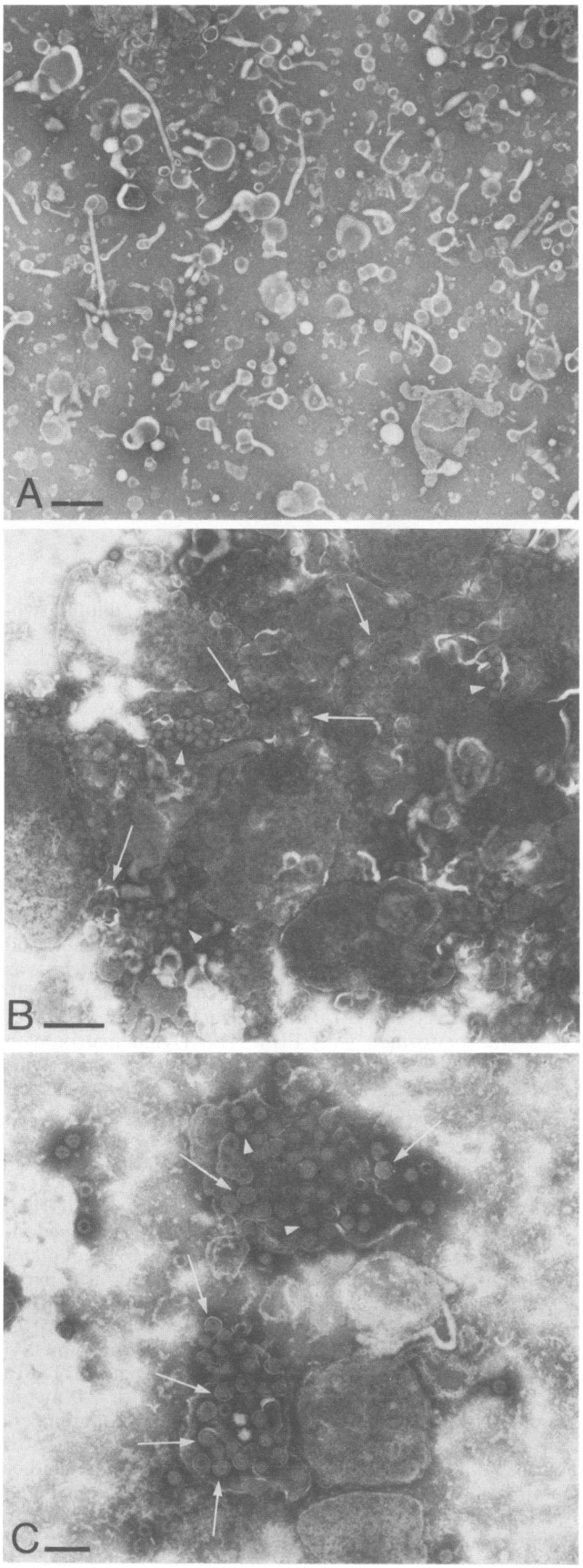


FIG. 1. Polyacrylamide gel analysis of bands derived from radiolabeled lysates of infected cells whose membranes were separated on a sucrose step gradient. Aliquots corresponding to the material at the 58%/68% (wt/vol) sucrose interface (band 1), the 44%/48% interface (band 3), the 39%/44% interface (band 4), the 33%/39% interface (band 5), and the sample/33% interface (band 6) were analyzed by SDS-PAGE alongside a radiolabeled SA11-infected cell lysate as a marker (SA11).

Cross-linking of proteins on immunoisolated enveloped virus particles and velocity gradient sedimentation. Aliquots of Dynal matrix with specifically bound enveloped virus particles adherent to their surfaces were resuspended in PBS. Freshly prepared cross-linker stock solutions (5 mg/ml) of either dithiobis(succinimidylproprionate) (DSP) in dimethyl sulfoxide or dithiobis(sulfosuccinimidylproprionate) (DTSSP) in water were added to a final concentration of 1.0 mg/ml (15). Samples were incubated with agitation at room temperature for 1 h and adjusted to 2 mM glycine. A portion of the cross-linked material was solublized in gel buffer either containing or lacking dithiothreitol (DTT) and analyzed by 11% SDS-PAGE. Another portion was solublized by boiling the Dynal in 1% TX-100 in MNT buffer, and the resultant supernatant was removed from the magnetic matrix



and was applied to the top of a 5 to 20% (wt/wt) sucrose gradient made in MNT buffer containing 0.1% TX-100 and centrifuged in an SW60 rotor at 53,000 rpm (300,000 × *g*) at 4°C. The runs were terminated in the range of $\omega^2 t$ equal to 0.6×10^{12} to 0.9×10^{12} rad^2/s (6 h). Nine-drop fractions were collected from the bottom of each tube with a 19-gauge needle, and the proteins of each or every other fraction were precipitated with acetone, solubilized in sample preparation buffer containing DTT, and boiled before analysis on an SDS-10% polyacrylamide gel.

Immunoprecipitation of VP7 from immunoisolated enveloped virus particles. Anti-VP7 polyclonal antiserum was raised in a rotavirus-free rabbit, kept in germfree isolation, against VP7 purified from an SDS-polyacrylamide gel. The rabbit was a generous gift from M. Estes, Baylor University, Houston, Tex. Aliquots of Dynal matrix with specifically bound enveloped virus particles at their surfaces were immunoprecipitated under denaturing or nondenaturing conditions as follows. Immunoprecipitations under denaturing conditions were performed by adding 150 μl of lysis buffer (1% TX-100, 1% deoxycholate, 0.15 M NaCl, 0.025 M Tris hydrochloride [pH 8.0], 0.1% SDS, 100 U of aprotinin per ml) to the Dynal aliquot and vortexing for 30 s before the addition of 50 μl of 2% deoxycholate–2% Nonidet P-40 in 1× PBS. The spheres were vortexed, and SDS was added to a final concentration of 2% before the mixture was boiled for 3 min. Buffer A (1 ml) consisting of 0.19 M NaCl, 0.005 M EDTA, 2.5% TX-100, 1 mg of BSA per ml, 0.001 M L-methionine, and 100 U of aprotinin per ml was added. Samples were then adjusted to 2.5% TX-100. The supernatants were removed from the Dynal beads and processed further as described below. For immunoprecipitation under nondenaturing conditions, 72A4, a monoclonal antiserum directed against the mature viral particle form of VP7 (11) (provided by A. R. Bellamy, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand), was used. Dynal samples were resuspended in 1% Nonidet P-40–0.15 M NaCl–0.05 M Tris-HCl (pH 7.2)–2 mM glucose–4 mM phenylmethylsulfonyl fluoride. To either the denatured or nondenatured sample, several microliters of the appropriate anti-VP7 antibody were added. In addition, 5 μl of a mixture of affinity-purified goat anti-mouse IgG, IgM, and IgA (Organon Teknika, Malvern, Pa.) was added for immunoprecipitation with the monoclonal antibody 72A4 to facilitate the binding of the latter antibody to the protein A-Sepharose CL4B beads (Pharmacia Inc., Piscataway, N.J.). Following the addition of protein A-Sepharose, samples were incubated for 2 to 3 h at 4°C on a rocking platform. Samples containing denatured antigens were washed by centrifugation three times with buffer B (0.15 M NaCl, 0.01 M Tris hydrochloride [pH 8.3], 0.1% TX-100, 0.005 M EDTA, 100 U of aprotinin per ml) and once with 1× PBS. Samples containing nondenatured antigens were washed twice in 1% Nonidet P-40–0.5% deoxycholate–0.1% SDS–

FIG. 2. Negative stain of aliquots of fractions from the sucrose gradient. The fraction corresponding to the 33%/39% (wt/vol) sucrose interface (band 5) (A) shows predominantly smooth-membraned saccules, tubules, and vesicles and a small amount of virus. The fraction corresponding to the 39%/44% and 44%/48% sucrose interfaces (bands 4 and 3, respectively) (band 3 shown here in both low [B] and high [C] magnification) shows many membrane elements of the ER containing both mature (arrowheads) and immature enveloped (arrows) virus. Bars A and B, 500 nm. Bar C, 200 nm.

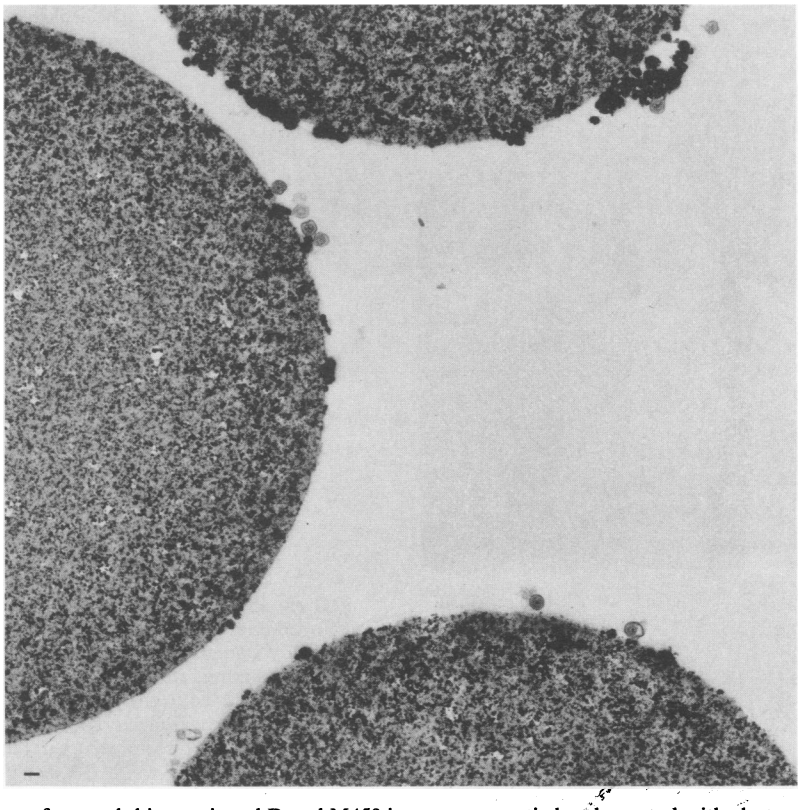


FIG. 3. Low magnification of several thin-sectioned Dynal M450 immunomagnetic beads coated with sheep anti-rabbit IgG and incubated with fractions corresponding to sheared band 4, which had affinity-purified NS28-β-galactosidase fusion protein antibodies added to it before Dynal incubaiton. Bar, 100 nm.

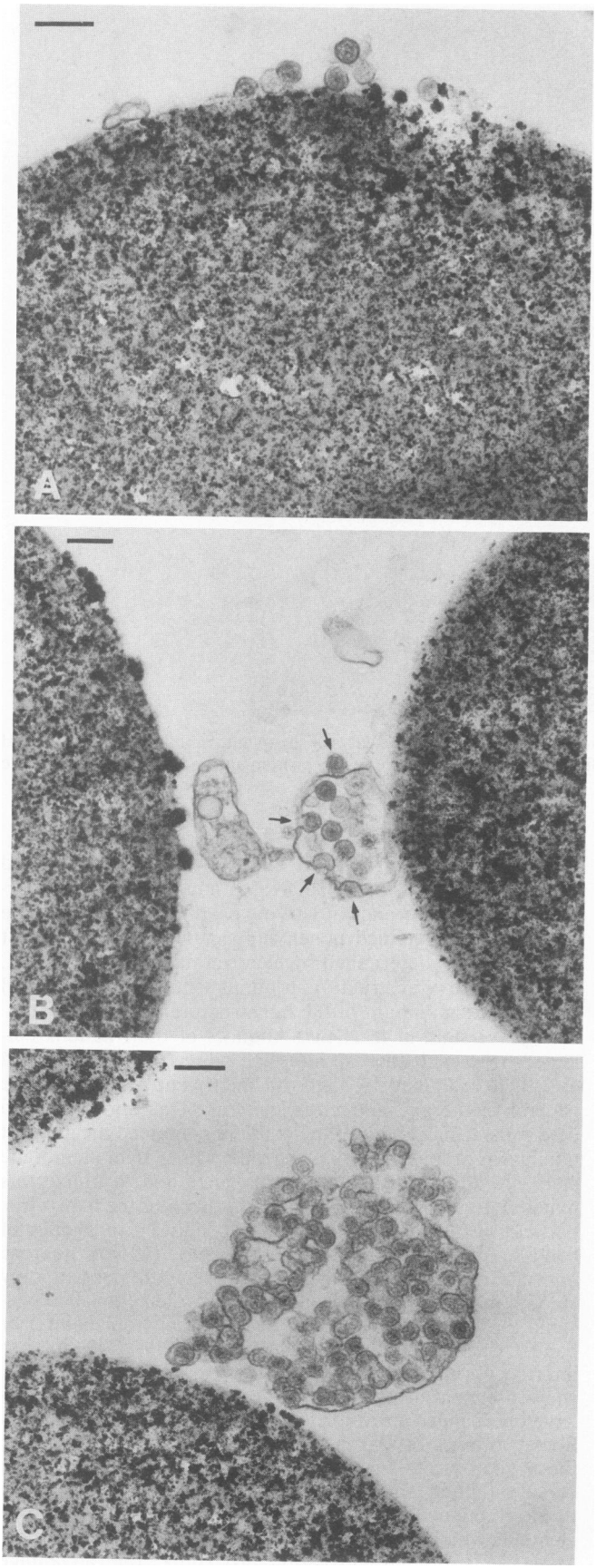
0.5 M NaCl–0.05 M Tris hydrochloride (pH 7.5) and once with 1× PBS before being analyzed by SDS-PAGE.

Protease treatment of immunoisolated enveloped virus particles. Dynal M450 particles with specifically bound immunoisolated material were resuspended in PBS and divided into three equal aliquots. The PBS was removed, and the beads were resuspended in 25 μl of 50 mM Tris buffer (pH 7.5) and 0.5 μl of 0.5 M $CaCl_2$, and then 0.5 μl of 0.15 M tetracaine was added to each sample to stabilize the membranes. Chymotrypsin and trypsin was added to samples with and without 1% TX-100 to a final concentration of 62.5 μg/ml. The third sample, the control, had no additions of protease or detergent. All three samples were incubated at 0°C for 60 min, and 100 μg of soybean trypsin inhibitor per ml, 200 U of aprotinin per ml, 5 mM ε-aminocaproic acid, 1 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride were added. Samples were analyzed by SDS-PAGE.

RESULTS

Fractionation of SA11-infected Ma104 cells. Rotavirus-infected Ma104 cells were radiolabeled, and the postnuclear supernatant was fractionated by sucrose density step gradient centrifugation to obtain a membrane fraction enriched in elements of the ER which contain virus. Distinct opaque bands appeared at each of the sucrose interfaces (band 1, 58%/68%; band 2, 48%/58%; band 3, 44%/48%; band 4, 39%/44%; band 5, 33%/39%; and band 6, sample/33%), corresponding to different membrane fractions. When aliquots of several of the bands were analyzed by SDS-PAGE, viral structural proteins VP1, VP2, VP4, VP6, and VP7 and nonstructural proteins NS53, NS35, and NS28 were apparent in each of the bands in varying proportions (Fig. 1). The fraction that contained nonenveloped mature virus almost exclusively, as determined by electron microscopy, band 1, contained the viral structural proteins with a relatively small contaminating amount of the nonstructural proteins.

Negative stain of fractions. Aliquots of the fractions collected from each sucrose interface were negatively stained and then examined by electron microscopy to assess the general membrane and virus content. This allowed us to determine which bands of the gradient contained the greatest number of profiles of virus-laden ER rather than membrane elements derived from other organelles and to utilize this material for further purification of the intermediate virus particle by the immunoisolation procedure. An aliquot of band 5, corresponding to the 33%/39% (wt/vol) sucrose interface (density, 1.14 g/ml), was seen to contain only smooth membrane vesicular and tubular elements and occasional profiles of virus (Fig. 2A). Material from band 3 or band 4, corresponding to the 44%/48% and 39%/44% sucrose interfaces, respectively (densities, 1.18 and 1.21 g/ml), showed numerous elements of the ER which contained both enveloped and mature forms of the virus (Fig. 2B and C). Some portions of disrupted mitochondria were present in these microsomal fractions as well. This was not a major concern though, since the antibody specific for NS28, added to the fractionated material, would only bind to membrane elements which displayed the NS28 protein on their surface. Thus, for the immunoisolation procedure, one advantage



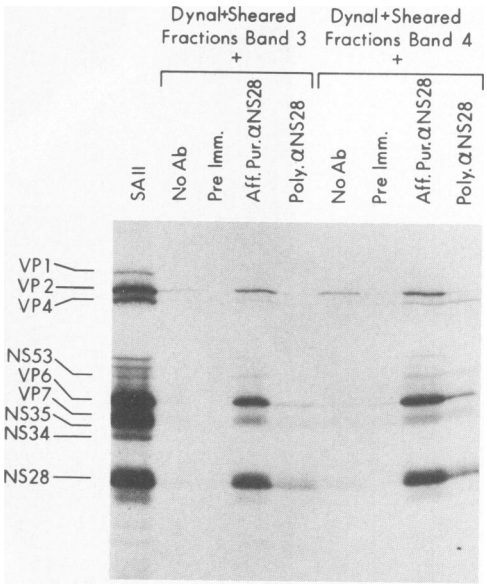


FIG. 5. Protein composition of the immunoisolated membrane-enveloped intermediate virus particles. The composition of material specifically bound to Dynal M450 spheres that had no antibody, preimmune serum, affinity-purified anti-NS28, or polyclonal antiserum to NS28 added to them was analyzed by SDS-PAGE following incubation with sheared sucrose gradient fractions corresponding to band 3 or band 4. The viral structural and nonstructural proteins are indicated.

was that the starting material need not be a purified fraction, merely one that is enriched in the elements of interest.

Electron microscopy of immunoisolated membrane-enveloped virus. Sheared membrane fractions incubated with either a polyclonal antiserum or an affinity-purified antibody to NS28 and Dynal particles showed many profiles of membrane-enveloped intermediate virus particles specifically bound along the surfaces of several spheres when examined by thin-section electron microscopy at a fairly low magnification (Fig. 3). Upon higher magnification, it was apparent that groups of the membrane-enveloped intermediate virus particles were the exclusive form of virus specifically bound to the Dynal surface (Fig. 4A) along with elements of membrane. One would expect this membrane to be derived from portions of the ER, since NS28 is inserted into it in a transmembrane orientation (3, 5). Also, portions of the ER with virus apparently in various stages of budding into it, and thus acquiring the transient membrane envelope, were specifically bound to the Dynal surface (Fig. 4B). When intermediate membrane-enveloped virus was immunoisolated with affinity-purified anti-NS28 from microsome-containing fractions derived from cells which had been treated with calcium-free medium and the ionophore A23187, even greater numbers of membrane-enveloped intermediate par-

FIG. 4. High magnification of the thin-sectioned Dynal surface showing several cross sections of specifically bound immunoisolated membrane-enveloped virus particles (A), a portion of the ER with membrane-enveloped virus (B), and virus in various stages of budding into the ER (B, arrows). (C) Cross section of a cluster of membrane-enveloped immunoisolated virus derived from infected cells treated with 5 μM ionophore A23187 in calcium-free medium for 3 h at 2 h postinfection. Bars, 200 nm.

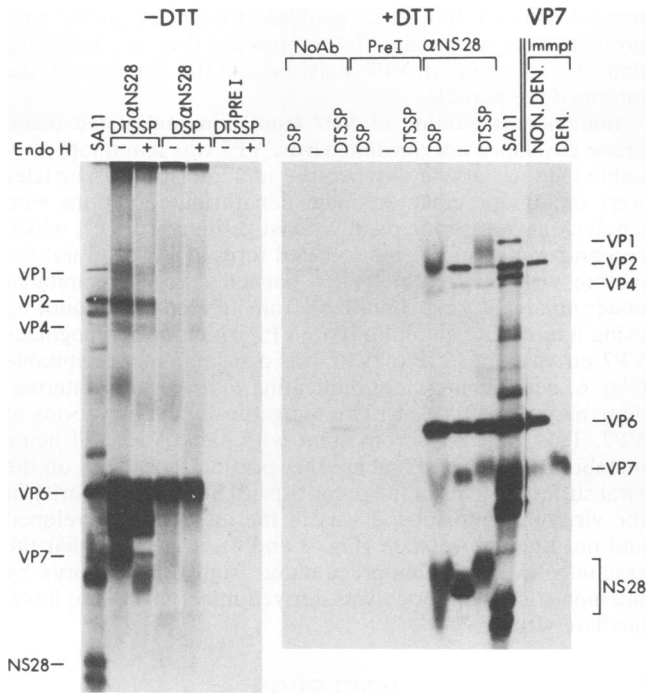


FIG. 6. Cross-linking of proteins of the immunoisolated membrane-enveloped virus particles and immunoprecipitation of the isolated particle with antibodies to VP7. Immunoisolated virus specifically bound to Dynal M450 was cross-linked with the thiol-cleavable cross-linker DSP (membrane permeable) or DTSSP (membrane impermeable). −, samples not treated with any cross-linker for samples which were reduced. Cross-linked samples were either not reduced (− DTT) or reduced (+ DTT) and, for the nonreduced species, digested with endo-β-*N*-acetylglucosaminidase H (endo H) for 1 h at 37°C before their analysis by SDS-PAGE. VP7 was immunoprecipitated (Immpt) from the immunoisolated virus particles under nondenaturing conditions (NON DEN) with the monoclonal antibody to VP7, which recognizes VP7 of mature virus, or under denaturing (DEN) conditions with the polyclonal antiserum to VP7, which recognizes the membrane-associated form of VP7. PreI, preimmune.

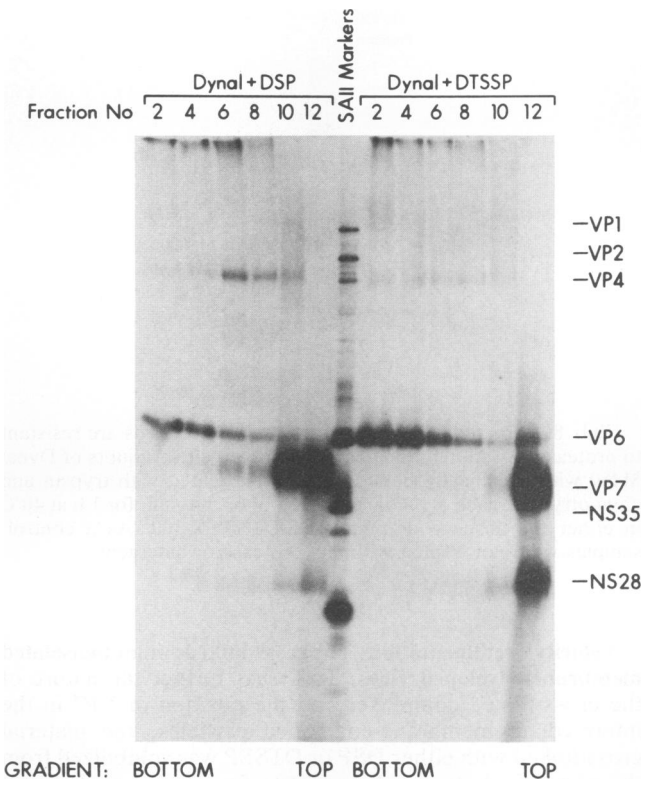


FIG. 7. VP7 on the immunoisolated virus intermediate is inaccessible to a membrane-inpermeable cross-linker. The proteins of membrane-enveloped intermediate virus particles specifically bound to the surface of Dynal M450 were cross-linked with either the membrane-permeable cross-linker DSP or the membrane-impermeable cross-linker DTSSP, and the solubilized complexes were separated by velocity sedimentation on a 5 to 20% (wt/wt) sucrose gradient centrifuged in the range of $\omega^2 t$ equal to 0.6×10^{12} to 0.9×10^{12} rad^2/s (6 h). Fractions were collected from the bottom of the tube and numbered. The precipitates of the proteins of the even-numbered fractions are displayed, and the viral proteins are indicated.

ticles were seen to be specifically bound to the Dynal surfaces (Fig. 4C). When preimmune antiserum from a rotavirus-free rabbit, or no antibody, was added to the fractions, this specific binding was not observed, and only occasionally random membrane elements and mature virus were observed nonspecifically associated with the Dynal.

Protein composition of immunoisolated membrane-enveloped intermediate virus. Infected cells were radiolabeled, and the immunoisolated membrane fractions were solublized and then analyzed by gel electrophoresis to determine the protein composition of the immunoisolated virus particles. It was observed that when either the affinity-purified anti-NS28 or polyclonal anti-NS28 was used, VP1, VP2, VP4, VP6, VP7, and NS28 were associated with the immunoisolated material (Fig. 5). The proportion of NS28 to VP7 was unique to this fraction and was increased in relation to the other viral proteins when compared with the infected-cell total lysate (Fig. 5). ER could not be the origin of the proteins present because VP7 would be present in the same amounts as NS28. The specific pattern was not seen in immunoprecipitation controls. If mature nonenveloped virus was the viral species bound to Dynal, NS28, the nonstructural glycoprotein, would not be present.

Cross-linking of immunoisolated membrane-enveloped intermediate virus. To examine the orientation and structural relationships between the various proteins, we cross-linked the immunoisolated material using two types of reagents. When material immunoisolated from normally infected cells was cross-linked with the thiol-cleavable membrane-permeable cross-linker DSP, and the material was not reduced prior to gel electrophoresis, NS28, VP7, and VP1 to VP4 were absent from the gel at their normal positions because they had been incorporated into higher-molecular-weight complexes (Fig. 6). However, when membrane-impermeable DTSSP was used, VP7, VP6, VP4, VP2, and VP1 were present in their normal positions on the gel and apparently were not cross-linked. Only NS28 was apparently cross-linked to a higher-molecular-weight (15) form running between VP4 and VP6 (Fig. 6). When the DSP-cross-linked complexed material was reduced with DTT, NS28, VP7, VP2, and VP4 were seen to reappear and to migrate in their normal positions on the gel (Fig. 6). When the DTSSP-treated sample was reduced, NS28 reappeared in its normal position as well (Fig. 6). These observations are consistent with only NS28 being accessible outside the membrane barrier.

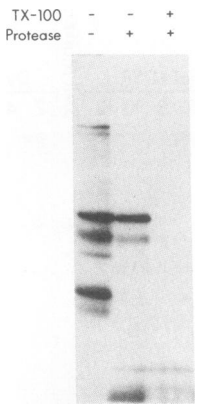


FIG. 8. VP6 and VP7 on the immunoisolated virus are resistant to protease digestion in the absence of detergent. Aliquots of Dynal M450 with specifically bound virus were treated with trypsin and chymotrypsin, each at a concentration of 62.5 μg/ml, for 1 h at 40°C in either the absence or presence of 1% TX-100. As a control, samples were not treated with any protease or detergent.

Velocity sedimentation of cross-linked immunoisolated membrane-enveloped virus. To assess further the nature of the cross-linked complexes and the position of VP7 in the intermediate membrane-enveloped particles, the material cross-linked with either DSP or DTSSP was solubilized from the magnetic matrix and applied to 5 to 20% (wt/wt) linear sucrose gradients before reduction. When the fractions from the gradient were reduced and analyzed by SDS-PAGE, it appeared that VP7 was cross-linked to some extent by the membrane-permeable cross-linker DSP (Fig. 7) such that VP7 was found in portions of the gradient of higher sucrose concentration (i.e., sedimenting as far as fraction 4), corresponding to higher-order complexes of VP7. This was apparently not the case for the membrane-impermeable cross-linker DTSSP (Fig. 7), with which all forms of VP7 were found nearer the top of the gradient, corresponding to monomeric forms of VP7. For the DSP-treated immunoisolated particles, VP4 appeared not only in fractions corresponding to its monomeric form (fractions 8 to 10) but also in fractions which corresponded to higher-molecular-weight oligomers of VP4 (fractions 2 to 4) or heterooligomeric complexes of VP4 and VP7 (fractions 6 to 8) derived from within the virus particle. The location of VP4 in fractions 2 to 6 in DTSSP-treated samples and the absence of VP7 implies that VP4 on the outside of the isolated particle can form homooligomers and does not cross-link with VP7 located within the enveloped particle. The paucity of NS28 present on the gradient most likely occurs because it has been chemically cross-linked to the antibody and in turn to the Dynal particle and is not released from the Dynal particle under the nonreducing conditions described. Material sedimented on the gradient was reduced just before gel electrophoresis.

Protease treatment of immunoisolated membrane-enveloped virus particles. To characterize further the positions of the viral structural and nonstructural proteins in the intermediate immunoisolated particle, we treated material specifically bound to the Dynal with protease in the presence or absence of detergent. When the immunoisolated virus particle was treated with protease in the absence of detergent, VP6 and VP7, and to some extent VP2, were seen to be protected from digestion (Fig. 8). However, when the particles were treated with TX-100 before protease treatment, all the viral proteins were digested with the protease (Fig. 8), indicating that the location of VP7 may be on the interior of the intermediate particle.

Immunoprecipitation of VP7 from immunoisolated membrane-enveloped intermediate virus. VP7 was immunoprecipitable (Fig. 6, last lane) when the immunoisolated particles were immunoprecipitated under denaturing conditions with a polyclonal antiserum made against gel-purified VP7, which recognizes a membrane-associated form of VP7 and not the mature viral VP7 form. When particles were precipitated under nondenaturing conditions from an identical aliquot by using a monoclonal antibody to VP7 which only recognizes VP7 on virus, a form of VP7 was also immunoprecipitable (Fig. 6, penultimate lane), indicating that the viral intermediate may exhibit at least two antigenically distinct forms of VP7. This is perhaps consistent with some VP7 still being membrane associated and another portion assembled on the viral surface as part of the outer capsid. Since the majority of the virus immunoisolated was in the membrane-enveloped and not the mature state (Fig. 3 and 4), it is likely that the amount of VP7 immunoprecipitated from isolated virus by the monoclonal antibody was derived mainly from the intermediate virus.

DISCUSSION

Rotaviruses are characterized by their unique mode of morphogenesis, in that virus production and maturation are restricted to the ER. The maturation process of rotavirus normally includes the passage of the virus particle through a membrane-encapsidated intermediate form within the ER lumen. The events and mechanism surrounding the maturation of this virus particle and the selective retention of outer capsid virus proteins and the loss of nonstructural proteins along with other membrane elements remain uncharacterized. We described a sequential sucrose step gradient fractionation procedure for infected cells which permitted the isolation of membrane fractions enriched in microsomes that contained membrane-enveloped virus particles within the ER lumen. Repeated shearing of these membrane elements resulted in the liberation of intermediate particles. The subsequent addition to the fractions of antibody directed against NS28 and then the immunomagnetic matrix allowed the separation of the ER and the membrane-enveloped virus from mature virus and other cellular organelles and membranes which did not exhibit NS28. The advantages of utilizing the inert magnetic matrix to isolate various cellular membrane domains have been documented by others (10), in that the spheres are easily resuspended and washed free of nonspecifically adherent material. In addition, the starting material can be a heterogeneous fraction from which one selectively immunoisolates organelles or membranes based on the specificity of the antibody reagents. The present study determined the protein composition of these immunoisolated membrane-enveloped virus particles, showing that the transmembrane nonstructural glycoprotein NS28 is indeed still detectable in these viral intermediates and that at least two antigenically distinct forms of VP7 are present as well. The immunoisolation of this subset of virus permitted the use of either a membrane-permeable or -impermeable cross-linking reagent to assess protein-protein interactions and the location of VP7 with respect to the transient membrane envelope of the virus. The membrane-permeable cross-linker was able to cross-link all the viral proteins except VP6, but the membrane-impermeable reagent only had an effect on NS28,

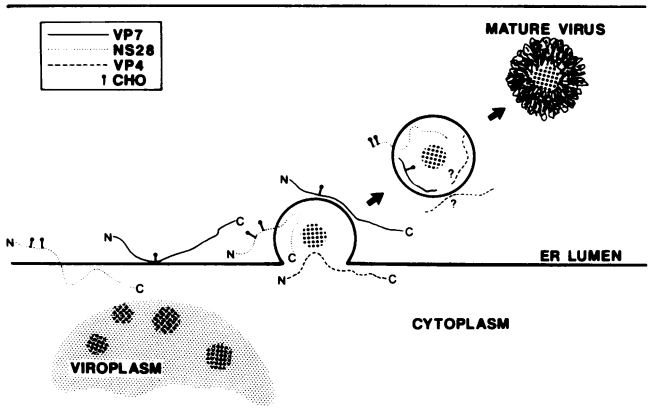


FIG. 9. Diagrammatic representation of the maturation of rotavirus and the putative location of the viral proteins with respect to the membrane. Single-shelled rotavirus particles bud into the ER, the membrane envelope is transiently acquired, and possible orientations of the nonstructural transmembrane glycoprotein NS28, the structural glycoprotein VP7, and the outer capsid protein VP4 are indicated.

a portion of which is located on the external side of the transient viral membrane. This result, taken together with that of the sedimentation study performed on cross-linked immunoisolated material, implied that VP7 had shifted its position from its luminal association with the ER membrane to the interior of the intermediate particle, since it could only be cross-linked by the membrane-permeable cross-linker (Fig. 9). This suggested that VP7 had relocated across the membrane envelope of the virus particle, possibly during the formation of the outer capsid by VP7 and VP4. Additional support is provided by the sedimentation and cross-linking results, which showed that VP7 and VP4 were located in the same fractions when only the membrane-permeable cross-linker was used (Fig. 7), suggesting that VP7 and VP4 form heterooligomeric complexes in the viral intermediate. It appears that during formation of the outer capsid and the rearrangement of VP7 and VP4, segments of VP4 within the virus particle may become exposed and thus accessible to protease and the membrane-impermeable cross-linker. The resistance of both VP7 and VP6, and to some degree VP2, to treatment of the immunoisolated virus particles with protease in the absence of detergent also supports the conclusion that they are inaccessible to protease and are located within the interior of the membrane-enveloped particle (Fig. 9) or in a conformation or complex indirectly rendering them inaccessible.

The isolation of the transiently occurring membrane-enveloped virus particles should enable examination of the energetic, ionic, and enzymatic requirements necessary for the uncoating process to occur in vitro, allowing us to extrapolate to events and mechanisms which might occur in vivo.

ACKNOWLEDGMENTS

We thank J. Rothblatt, A. R. Bellamy, and R. Trimble for their generous gifts of antibodies and enzymes. The critical discussion and reading of the manuscript by David Maass and the expert technical assistance of R. P. Kawakami and J. Lee for the growth and propagation of the SA11 rotavirus are greatly appreciated.

This work was supported by Public Health Service grant R01-CA13402-17 and core cancer grant PO1-CA13330 from the National Institutes of Health, awarded to P.H.A.

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