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Rotavirus SA11 Genome Segment 11 Protein Is a Nonstructural Phosphoprotein

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We investigated properties of the rotavirus genome segment 11 protein. A rotavirus SA11 genome segment 11 cDNA which contains the entire coding region was sequenced and inserted into the baculovirus transfer vector pVL941. Recombinants containing gene 11 cDNA were selected, and the gene 11 product expressed in *Spodoptera frugiperda* cells infected with these recombinants was inoculated into guinea pigs to produce hyperimmune antiserum. Characterization of the antiserum showed that it recognized a primary translation product with a molecular weight of 26,000 (26K protein) in recombinant-infected insect cells, in SA11-infected monkey kidney cells, and in cell-free translation reactions programmed with SA11 mRNA. A modified 28K product was also detected but only in SA11-infected monkey kidney cells. The 26K and 28K proteins were shown to be phosphorylated in infected monkey kidney cells, and the 26K protein was phosphorylated in insect cells. We were unable to identify what type of modification caused the molecular weight shift to 28,000 in infected monkey kidney cells. Large amounts of the gene 11 product were detected by immunofluorescence in discrete foci in the cytoplasm of infected monkey kidney cells. Viruses of all known serotypes were also detected by immunofluorescence by using hyperimmune antiserum to the SA11 gene 11 product. The antiserum reacted with particle-depleted cytosol fractions but did not react with purified virus particles by immunoprecipitation or immunoblotting; it also did not neutralize virus infectivity in plaque reduction neutralization assays. Therefore, we conclude that the primary gene 11 product is a nonstructural phosphoprotein which we designated NS26.

Rotaviruses are members of the *Reoviridae* family with a double-shelled protein capsid and a genome of 11 segments of double-stranded RNA (11, 14). Protein-coding assignments for each genome segment have been established on the basis of identification of the protein(s) synthesized from either double-stranded RNA or mRNA in cell-free translation systems. The structural or nonstructural status of 10 of the 11 gene products was determined by comparing the translation products with proteins in purified virus particles and in virus-infected cell lysates (1, 4, 6, 7, 20, 23, 27, 32). Such studies have clearly shown that simian rotavirus SA11 genome segments 1, 2, and 3 encode core proteins, segment 6 encodes the inner capsid protein, segments 4 and 9 encode outer capsid proteins, and segments 5, 7, 8, and 10 encode nonstructural proteins. The protein product of genome segment 11 was identified as a protein with an apparent molecular weight of ~26,000 (26K protein) when purified gene 11 mRNA was translated in rabbit reticulocyte lysates (RRL) and in wheat germ (WG) lysates (4, 6, 7, 23), and this protein was shown to be processed to a 28K band in infected cells in pulse-chase experiments (7). Although the primary translation product was identified, its designation as a structural or nonstructural protein has remained controversial. For example, the gene segment 11 protein was tentatively assigned as a nonstructural glycoprotein (1), a structural protein of the inner virus shell (27), a minor outer capsid protein (24), or a minor neutralizing antigen (25). Antibody made to a denatured 26K protein from SA11-infected cells was reported to have neutralizing activity (2). In an independent electron microscopic immunocytochemical study, antibody which reacted with the gene 11 protein and was made to denatured 26K protein isolated from sodium dodecyl sulfate (SDS)-polyacrylamide gels was found to react with viroplasms in SA11-infected monkey kidney cells (30). However, technical limitations (lack of a high-titered monospecific antiserum and lack of sufficient protein for direct characterization) prevented definitive proof of whether minor amounts of the gene 11 product were present in virus particles. We have pursued this question, because the presence of a third minor outer capsid protein in rotavirus particles could be important for understanding the biology of these viruses and for ongoing vaccine development programs. This report describes studies that used the baculovirus expression system to synthesize the gene 11 protein and antiserum produced to this expressed protein to characterize and clarify the properties of the SA11 gene 11 product.

MATERIALS AND METHODS

Viruses and cells. The rotaviruses used in this study were simian rotavirus SA11, clone 3 (serotype 3), human Wa (serotype 1), human S2 (serotype 2), porcine Gottfried (serotype 4), porcine OSU (serotype 5), bovine Nebraska calf diarrhea virus (serotype 6), and human 69M (serotype 8). These rotaviruses were pretreated with 10 μg of trypsin per ml and propagated in fetal rhesus monkey kidney (MA104) cells as described previously (9). Wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant virus pVL941/SA11-11 were propagated and assayed in confluent monolayers of *Spodoptera frugiperda* cells (IPLB-SF21-AE) at 27°C in Grace medium as previously described (8).

Preparation and sequencing of gene 11 cDNA. A pBR322 plasmid containing a full-length cDNA of SA11 gene 11 was synthesized from genomic RNA by addition of poly(A) tails as previously described (10). The cDNA was excised from *Eco*RI and *Hin*dIII sites and subcloned into transcription

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vector pBS (Stratagene Cloning Systems, La Jolla, Calif.). Tails at the 5′ end of the cDNA were removed by restriction enzyme digestion at a *Hin*pI (*Hha*I) site 9 base pairs upstream from the first ATG. This *Hin*pI construct was sequenced by the Sanger dideoxy-chain termination method. The sequence was determined from both directions by using both T3 and T7 primers and Sequenase (United States Biochemical Co., Cleveland, Ohio) as recommended by the manufacturer. The sequence of the middle section of the gene was confirmed by sequencing an internal *Rsa*I construct.

Selection of baculovirus recombinants containing gene 11 cDNA inserts and preparation of antiserum to the expressed protein. Gene 11 cDNA was inserted into the *Bam*HI site of baculovirus transfer vector pVL941 (21), kindly provided by Verne Luckow and Max Summers, Texas A&M University, College Station, Tex. The gene 11 cDNA was excised from pBS at the *Eco*RI and *Hin*dIII sites, and after filling in of both the gene 11 cDNA and the *Bam*HI-digested pVL941 with deoxynucleoside triphosphate with DNA polymerase I (Klenow fragment), the cDNA fragment was blunt end ligated to dephosphorylated pVL941 with T4 DNA ligase. After transformation of *Escherichia coli* (DH5a; Bethesda Research Laboratories, Gaithersburg, Md.), plasmids in recombinant ampicillin-resistant colonies were screened by restriction enzyme analysis for inserts in the correct transcriptional orientation. One such clone, designated pVL941/SA11-11, was used to derive recombinants following cotransfection of *S. frugiperda* cells with CsCl gradient-purified wild-type AcNPV DNA by using the calcium phosphate precipitation procedure as previously described (8, 33). Gene 11 recombinant viruses were selected by identifying occlusion-negative plaques and were confirmed by hybridization with a gene 11 probe. Virus in occlusion-negative plaques was plaque purified four times and then used to prepare virus stocks. The resulting gene 11 recombinant was used to prepare expressed gene 11 protein to generate hyperimmune antisera in guinea pigs and mice.

Monospecific antiserum to expressed gene 11 protein was prepared in two rotavirus-negative guinea pigs (Elm Hill Breeding Farms, Chelmsford, Mass.) and four inbred BALB/c mice. Each guinea pig was inoculated intramuscularly three times with 3.18 mg of total protein of the cytosol and membrane fractions obtained from *S. frugiperda* cells infected with recombinant pVL941/SA11-11 virus. The first immunization was in complete Freund adjuvant, and the two subsequent immunizations were with incomplete Freund adjuvant. Mice were inoculated intraperitoneally with the cytosol fraction. Sera were collected 7 days after the last injection and were tested for the presence of antibodies by immunoprecipitation and immunofluorescent-antibody staining with mock-infected and SA11-infected cells. Hyperimmune serum was adsorbed with mouse liver powder and wild-type AcNPV-infected *S. frugiperda* cells before being used in experiments.

Preparation of radiolabeled rotavirus proteins synthesized in infected cells and in cell-free systems. To prepare SA11-infected MA104 cell lysates, confluent monolayers of MA104 cells (1×10^6 to 2×10^6 cells per 35-mm-diameter well) were infected with trypsin-activated SA11 virus at a high multiplicity of infection (20 to 30 PFU per cell) per milliliter in 199 medium containing 5 to 7 μg of actinomycin D (Pharmacia LKB Biotechnology, Piscataway, N.J.) per ml and 1 mg of tunicamycin (TM; Sigma Chemical Co., St. Louis, Mo.) per ml if indicated (7). Mock-infected cells were prepared concurrently as previously described (7). Thirty minutes before being labeled with [^{35}S]methionine (40 μCi/ml; Tran^{35}S label; ICN Pharmaceuticals Inc., Irvine, Calif.), cells were starved in methionine-free minimal essential medium containing 5 to 7 μg of actinomycin D per ml for 30 min. For phosphate labeling, the cells were fed with phosphate-free minimal essential medium at 2 h postinfection and labeled with 400 μCi of $^{32}P_i$ (8,100 Ci/mmol; carrier free; ICN) per ml at 5 h postinfection. For sugar labeling, the cells were fed with glucose-free minimal essential medium after 1 h of adsorption and labeled with 100 μCi of [^{3}H]galactose or [^{3}H]glucosamine per ml. After an appropriate labeling period, the cells were harvested in 300 ml of either RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris hydrochloride [pH 7.2], 1% Trasylol) or 0.5 M NaCl–0.5% Nonidet P-40, sonicated for 1 min, and pelleted in a microcentrifuge. For pulse-chase experiments, cells were labeled for 10 min and then chased for the indicated length of time with 400× cold methionine and 50 μg of cycloheximide (Sigma) per ml.

To prepare radiolabeled SA11 virus particles, roller bottles of MA104 cells (5×10^7 cells per roller bottle) were washed with 199 medium without serum. Virus stocks were treated with trypsin, and cells were infected with high multiplicities (20 to 30 PFU per cell) of SA11. After adsorption, monolayers were washed to remove residual trypsin, and amino acid-free Eagle minimal essential medium containing 20 μCi of a ^{3}H-labeled L-amino acid mixture (ICN) per ml was added. Labeled virus was harvested when a complete cytopathic effect was observed. The procedures used for virus purification, preparation of rotavirus mRNA, and production of virus-encoded proteins in RRL and WG lysate have been described previously (24).

Immunoprecipitation and analyses of protein products by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels were performed by the procedures described by Ericson et al. (7). Radiolabeled proteins were monitored on gels following fluorography as described by Bonner and Laskey (3).

Phosphatase treatment. To demonstrate that phosphorylation was specific for the gene 11 protein, ^{32}P-labeled immunoprecipitates were treated with *E. coli* bacterial alkaline phosphatase (13). Briefly, immunoprecipitates were suspended in 15-μl reaction mixtures containing 20 mM Tris (pH 8.0) and 7 μg of alkaline phosphatase (Sigma) and were incubated at 37°C for 2 h. After incubation, 2× sample buffer was added immediately, followed by electrophoresis on a 12% polyacrylamide gel. Phosphatase digestion of purified histone H1 was monitored as a positive control.

Immunoblotting analysis. Purified SA11 double-shelled particles, radiolabeled SA11 lysates, and immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane in Tris-glycine-methanol buffer; the membrane was blocked with BLOTTO (18) for 2 h and then reacted with primary antibody (guinea pig or mouse serum raised against gene 11 recombinant protein or gene 10 protein) in a 1:10 dilution of BLOTTO overnight. The detector antibody was alkaline phosphatase-labeled goat anti-guinea pig immunoglobulin G or goat anti-mouse immunoglobulin G (HyClone Laboratories, Logan, Utah), and the color reaction was completed in Tris buffer (pH 10) containing *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt as described elsewhere (4a).

Preparation of subcellular fractions. *S. frugiperda* cells seeded in 75-cm^2 flasks were infected with recombinant

baculovirus (pVL941/SA11-11; 20 PFU per cell) in Grace medium. Infected cells were labeled with 40 μCi of [^{35}S]methionine per ml for 1 h at 24, 48, and 72 h postinfection. MA104 cells were infected with SA11 rotavirus as described previously and harvested at 6 to 7 h postinfection.

To prepare different subcellular fractions, cells were removed from the flasks with a rubber policeman, pelleted, washed twice in phosphate-buffered saline (pH 7.4), swollen in 1 mM $MgCl_2$, and disrupted by Dounce homogenization. The nuclei were pelleted at 1,100 × *g* for 5 min and washed twice with 20 mM Tris buffer (pH 5.5) containing 0.5% sodium deoxycholate and 1% Nonidet P-40 and once with 1 mM $MgCl_2$. The supernatant fluid from which nuclei were pelleted (clarified at 21,500 × *g* for 30 min) was designated the cytosol fraction, whereas the pellet was designated the membrane fraction. The cytosol fraction was further divided into two parts. One of these was clarified at 186,000 × *g* for 1 h (particle depleted). The membrane fraction was further fractionated through a 20 to 50% discontinuous sucrose gradient. For immunization, *S. frugiperda* cell fractions were prepared in the same manner but without addition of [^{35}S]methionine. The cytosol fraction was concentrated and used alone or suspended with the membrane fraction in a small volume for immunization.

Indirect immunofluorescent-antibody staining. Confluent monolayers of MA104 cells grown on 10-mm-diameter cover slips were infected with rotaviruses of seven serotypes. Cover slips were harvested between 7 and 24 h postinfection, depending on the virus strain. Cover slips were rinsed once with phosphate-buffered saline (pH 7.4), fixed in 100% ethanol, and air dried. For indirect immunofluorescent-antibody staining, cover slips were rehydrated with phosphate-buffered saline, reacted with a 1:20 dilution of antiserum in a humid chamber at 37°C for 30 min, washed in phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated goat anti-guinea pig serum at a 1:40 dilution (9). Cover slips were examined with a Zeiss epifluorescence microscope.

RESULTS

Nucleotide sequence of SA11 genome segment 11. The nucleotide sequence and deduced amino acid sequence of SA11 genome segment 11 are shown in Fig. 1. The cloned copy of gene 11 was 667 bases long and contained the conserved 3′-terminal sequence found in other rotavirus genome segments (Fig. 1A; M. K. Estes, *in* B. N. Fields, ed., *Virology*, in press). Because of the reconstruction of the cDNA, the 5′ end of the gene was missing and only nine base pairs upstream from the first ATG were retained. The sequence at the 5′ end of the gene was obtained by RNA sequencing. The longest open reading frame (ORF) encodes a protein of 198 amino acids with a calculated molecular weight of 21,725 (Fig. 1B). A second in-phase methionine is found at amino acid 52 and, if used, would produce a protein of 148 amino acids with a calculated molecular weight of 16,252. An out-of-phase ORF, which could potentially code for a protein of 92 amino acids with a calculated molecular weight of 11,012, is also present in the sequence. The protein from the longest ORF is predicted to be highly hydrophilic, slightly basic (+1 charge at neutral pH), and very rich in serine (19.2%) and threonine (4.6%). Comparison of our nucleotide sequence for SA11 clone 3 genome segment 11 with the published sequences of other rotavirus strains showed 88.7% homology between SA11 and bovine UK

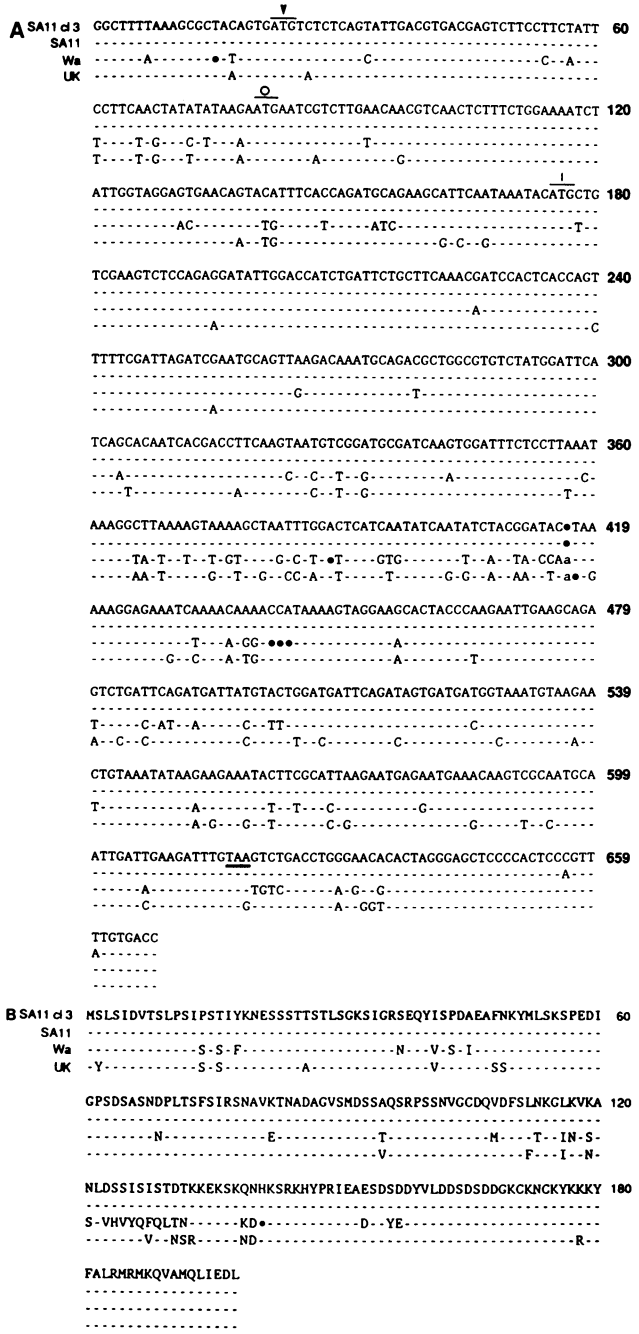

FIG. 1. Nucleotide and deduced amino acid sequences of SA11 genome segment 11. (A) Nucleotide sequence of the cDNA of SA11 clone (cl) 3 genome segment 11. Because of subcloning of the cDNA, the first 12 nucleotides were obtained by RNA sequencing. The published sequences of three other rotavirus genes 11 (SA11 [27], Wa [17], and UK [35]) are included for comparison. The positions of the first initiation codon (▼), a second in-phase potential initiation codon (I), an out-of-phase potential initiation codon (O), and the stop codon (underlined) are indicated. Uppercase letters indicate aligned nonidentical bases; lowercase letters indicate unaligned bases. (B) Deduced amino acid sequence of SA11 rotavirus gene 11. The longest ORF contains 198 amino acids; variation in the amino acid sequence with the published sequences for other rotaviruses (17, 28, 36) is shown. Dashes indicate aligned identical bases or amino acids. Dots indicate gaps in the nucleotide or amino acid alignment. A potential mononucleotide-binding sequence (20) is found between Gly-31 and Ser-37.

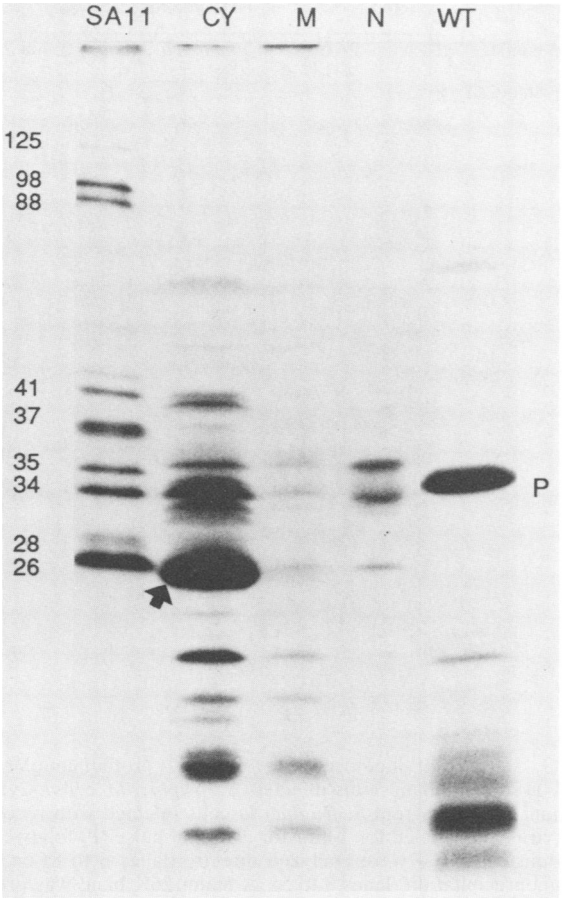

FIG. 2. Synthesis of the gene 11 protein in recombinant virus pVL941/SA11-11-infected *S. frugiperda* cells. Confluent monolayers of *S. frugiperda* cells were infected with recombinant virus at a multiplicity of infection of 20 PFU per cell. Infected cells were labeled with [^{35}S]methionine for 1 h at 48 h postinfection. Cells were harvested and fractionated into cytosol (CY), membrane (M), and nuclear (N) fractions as described in Materials and Methods. An SA11-infected MA104 cell lysate (SA11) which served as a control is shown on the left. The molecular weight (in thousands) of each virus protein is indicated on the left. The polyhedrin (P) protein of wild-type (WT) baculovirus is shown on the right. A large quantity of 26K protein (⬟) was found mainly in the cytosol fraction.

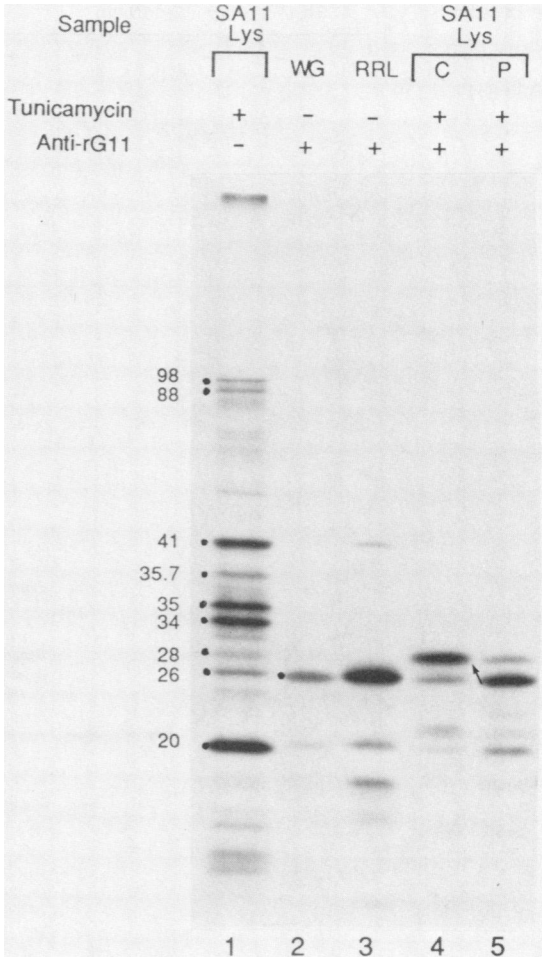

FIG. 3. Identification of gene 11 protein(s) made in cell-free translation systems and in SA11-infected cells. WG lysate and RRL were programmed with SA11 total mRNA, and [^{35}S]methionine-labeled proteins were precipitated with anti-rG11 serum. The proteins in SA11-infected MA104 cells were labeled with [^{35}S]methionine for 10 min (P) in the presence of TM (1 μg/ml) and then chased with cold methionine for 40 min (C). All proteins labeled in the TM lysate in the pulse (lane 1) and those precipitated with anti-rG11 serum from the pulse and chase (lanes 4 and 5) are compared. The modified 28K protein is evident in the chase sample (lane 4, arrow). Molecular weights in thousands are given on the left.

rotaviruses (36), 81.1% homology between SA11 and human Wa rotaviruses (17), and 99.7% homology between our SA11 sequence and another one published recently (28). The homologies between the deduced amino acid sequence of our SA11 and those of the UK, Wa, and other SA11 rotavirus proteins were 90.9, 83.8, and 100%, respectively.

Expression of the gene 11 protein using a baculovirus recombinant. The pVL941 transfer vector was constructed to provide high-level expression of nonfusion foreign proteins (21). Expression of the [^{35}S]methionine-labeled gene 11 protein was easily detected at 36 h postinfection in *S. frugiperda* cells infected with pVL941/SA11-11 recombinant virus. The expressed protein was also observed at 48 h postinfection in an SDS-polyacrylamide gel stained with Coomassie brilliant blue. The expressed protein constituted about 5% of the total cellular protein made. Cell fractionation experiments showed that the expressed protein was found mainly in the cytosol fraction (Fig. 2).

Characterization of antisera against the expressed gene 11 protein. A mixture of cytosol and membrane fractions from pVL941/SA11-11 recombinant virus-infected *S. frugiperda* cells was used to immunize two guinea pigs. Preimmune and hyperimmune sera were characterized by immunoprecipitation with proteins made in cell-free translation systems and in mock- or virus-infected mammalian or insect cells. Preimmune sera did not immunoprecipitate translation products from RRL and WG lysate reactions programmed with the SA11 viral mRNA, SA11-infected MA104 cell lysates, or AcNPV-infected *S. frugiperda* cell lysates (data not shown). Hyperimmune (designated as anti-rG11) serum did not react with mock-infected MA104 cell lysates or with translation reactions from RRL or WG lysate made without specific viral mRNA (data not shown).

Anti-rG11 serum precipitated a major protein with a molecular weight of 26,000 (26K protein) from RRL and WG lysate reactions programmed with total mRNA of SA11 (Fig.

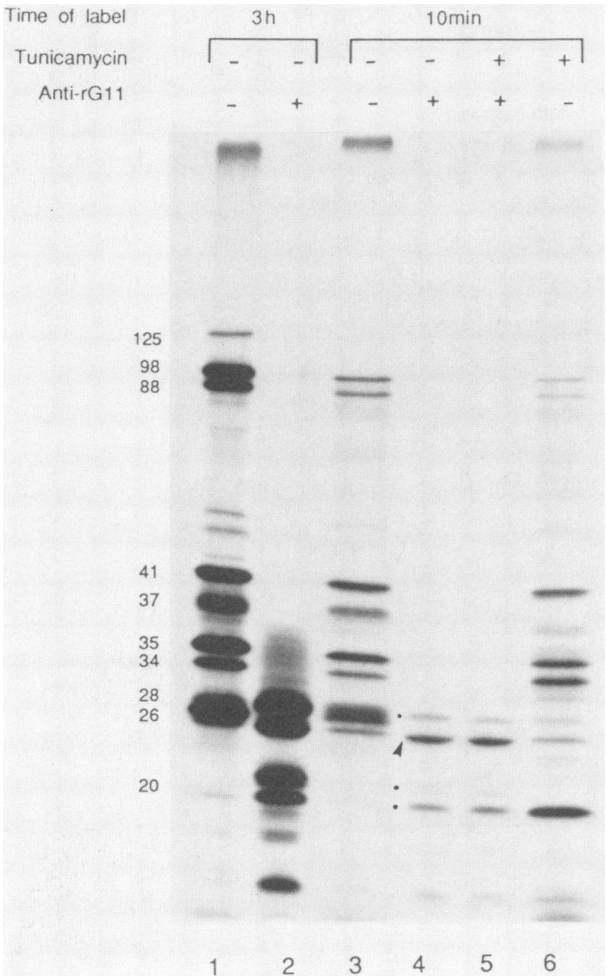

FIG. 4. Detection of gene 11 protein(s) in MA104 cells by immunoprecipitation. The polypeptides in MA104 cells infected with SA11 rotavirus were labeled with [^{35}S]methionine at 4 h postinfection for 3 h (lanes 1 and 2) or at 6.5 h postinfection for 10 min (lanes 3 to 6) with or without 1 μg of TM per ml. The predominant protein band that reacted with hyperimmune anti-rG11 serum is marked by an arrowhead, and minor bands are marked by dots. It remains unknown whether the bands below the 26K band are from translation at the second in-phase or out-of-phase initiation codon, premature termination, or proteolytic breakdown.

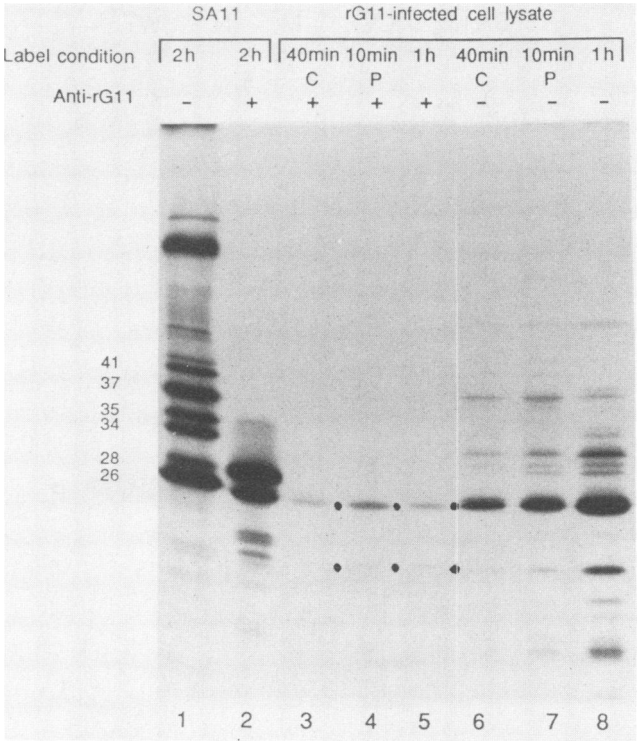

FIG. 5. Lack of modification of gene 11 protein in pVL941/SA11-11 recombinant virus-infected *S. frugiperda* cells. Cytosol fractions prepared from *S. frugiperda* cells infected with recombinant virus and labeled for 1 h or for a 10-min pulse (P) followed by a 40-min chase (C) were analyzed directly (lanes 6 to 8) or after immunoprecipitation (lanes 3 to 5). A major 26K band was precipitated with gene 11 antiserum, and this band comigrated with the 26K protein precipitated from mammalian cells infected with rotavirus SA11 (lane 2). Molecular weights in thousands are given on the left.

3). Bands of 26K and 28K proteins were detected in pulse-labeled SA11-infected MA104 cell lysates, and the 26K band was the predominant one (Fig. 3). In SA11-infected MA104 cells, after a 40-min chase with 400× cold methionine, the 26K band was processed to a higher-molecular-weight 28K band (Fig. 3). These data indicated that a posttranslational modification occurred. No difference in the migration pattern or distribution of the bands was observed when metabolic labeling was performed with or without TM (Fig. 4, lanes 4 and 5). Similarly, a major 26K band was detected in cytosol fractions prepared from pVL941/SA11-11 virus-infected *S. frugiperda* cells after a 10-min pulse or 1 h of labeling (Fig. 5, lanes 4 and 5). However, the protein patterns in the *S. frugiperda* cells differed from those in MA104 cells, since no molecular weight shift was observed after a 40-min chase in the insect cells (Fig. 5, lane 3).

Some lower-molecular-weight bands (20K, 22K, and smaller) were also precipitated from infected cells or cell-free translation reactions (Fig. 3 to 5). However, the 20K and 22K and other lower-molecular-weight bands were not detected consistently in immunoprecipitation assays. They were seen clearly when samples were labeled for long times (3 h; Fig. 4) or when fluorographs were exposed for long periods; they were not seen when immunoprecipitations were performed with mouse antiserum made to the expressed gene 11 protein.

The 20K and 22K bands (related to the gene 11 protein) in infected MA104 cells were distinguished from the 20K band detected in infected cells treated with TM and known to be the precursor of the 28K glycoprotein encoded by genome segment 10 (7). This was determined by performing immunoblots of the proteins immunoprecipitated with hyperimmune anti-rG11 serum immobilized on protein A-Sepharose (Pharmacia, Uppsala, Sweden). The membranes were probed with either guinea pig or mouse hyperimmune serum to the gene 11 product or to the gene 10 protein. Hyperimmune serum to the genome segment 10 protein (NS28) detected a 20K band only in TM-treated lysates and not in immunoprecipitates prepared with anti-rG11 serum (data not shown).

Localization of the gene 11 protein in MA104 cells. Anti-rG11 serum was used to localize the protein by immunofluorescence. A distinct immunofluorescence pattern was ob-

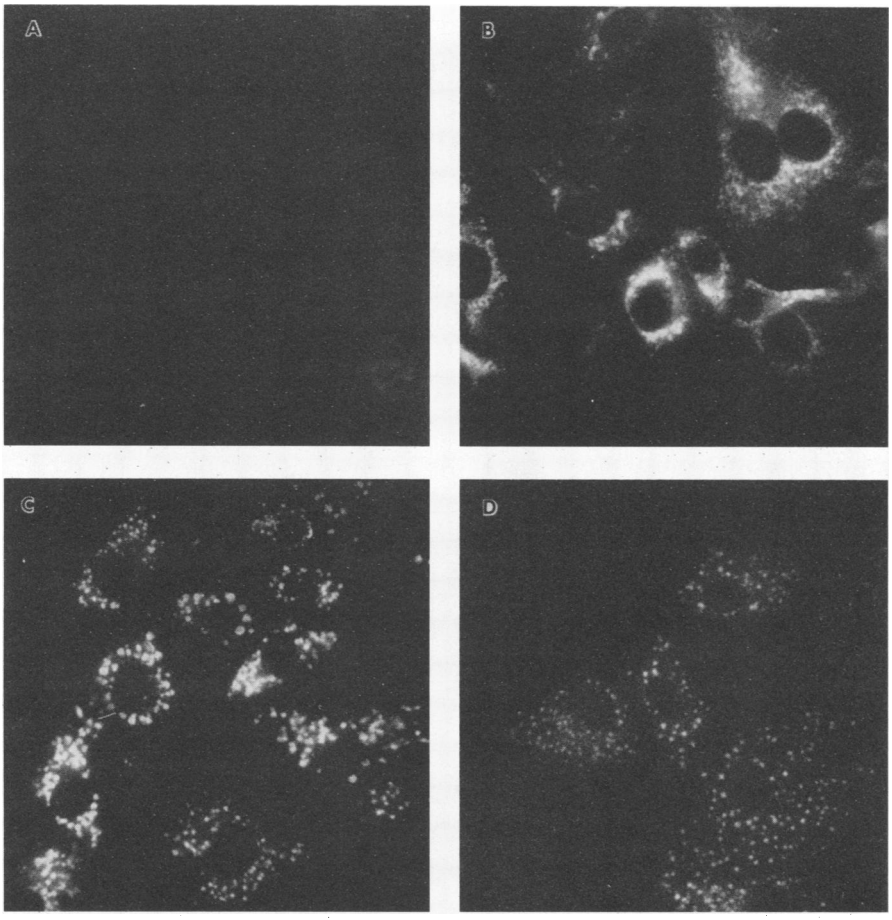

FIG. 6. Localization of gene 11 protein in cells by indirect immunofluorescence. (A) Mock-infected monkey kidney (MA104) cells reacted with hyperimmune antiserum to the baculovirus-expressed gene 11 protein (rG11); (B) SA11-infected MA104 cells reacted with hyperimmune serum to double-shelled SA11 virus; (C) SA11-infected MA104 cells reacted with hyperimmune anti-rG11 serum; (D) porcine (OSU) rotavirus-infected MA104 cells reacted with anti-rG11 serum. Each serum was tested at a 1:20 dilution followed by fluorescein-conjugated goat anti-guinea pig immunoglobulin at a 1:40 dilution. The gene 11 protein is seen in discrete foci (probably viroplasms) throughout the cytoplasm.

served only in the cytoplasm of SA11-infected cells (Fig. 6). The punctate staining pattern observed was seen in cells infected with viruses of each of seven rotavirus serotypes. The distribution of protein in subcellular fractions from ^{35}S-labeled SA11-infected MA104 cells was also examined. Membrane, cytosol, and nuclear fractions were prepared, and the membrane fraction was further fractionated through a discontinuous (20 to 50%) sucrose gradient. Each of six membrane fractions, the cytosol, and the nuclei were tested for reactivity with the hyperimmune serum (data not shown). The gene 11 proteins were found only in the cytosol and clarified cytosol fractions (Fig. 7).

Gene 11 protein(s) is a nonstructural protein. The previous experiments showed major 26K and 28K bands (and some minor bands) in infected cells reacted with anti-rG11. The mechanism by which the bands (other than the 26K primary product) originated was not identified in this study, but the antiserum was useful for determining whether any of these bands was present in purified virus particles. ^{3}H-amino-acid-labeled SA11 virus particles purified in gradients of metrizamide (which allows better retention of the outer-capsid proteins) were shown to contain a minor amount of an ~28K protein band (data not shown) when analyzed by SDS-polyacrylamide gel electrophoresis. This preparation of particles (when tested either directly or after boiling of the preparation in RIPA buffer to disrupt virus particles) did not react with the anti-rG11 serum in immunoprecipitation (Fig. 7, lane 6) and immunoblotting assays (data not shown). To further determine whether the gene 11 protein(s) was present in the cytosol but was not associated with viral particles, a cytosol fraction was clarified at 186,000 $\times$ g for 1 h. The supernatant fluid was carefully removed, and the supernatant was used for immunoprecipitation; the gene 11 protein was precipitated only from the supernatant. Hyperimmune antiserum prepared to double-shelled particles (containing ~28K bands) also did not precipitate the gene 11 protein from infected cells. Anti-rG11 serum was tested for virus-neutralizing activity in a plaque reduction neutralization test. No detectable neutralizing activity was found when the serum (1:10 dilution) was tested against 50 to 100 PFU of SA11.

Gene 11 protein(s) is a phosphoprotein. Pulse-chase experiments showed that the gene 11 protein underwent posttranslational modification. The predicted amino acid sequence indicated that this protein is rich in serine and threonine; therefore, the increase in molecular weight could be due to posttranslational modification(s) by addition of carbohydrate or phosphate moieties. To examine these possibilities, SA11-

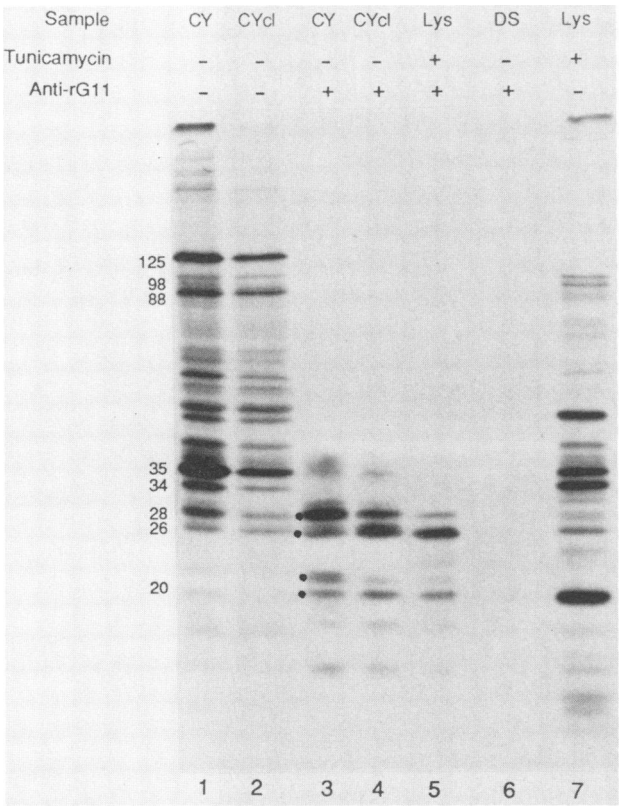

FIG. 7. Reactivity of gene 11 antiserum with protein(s) in MA104 cells following cell fractionation or in purified virus particles. ^{35}S-labeled proteins in SA11-infected MA104 cells were separated (186,000 × *g*) into cytosol (CY) and clarified cytosol (CYcl) (particle-depleted) fractions. Proteins labeled in a 10-min pulse in TM-treated SA11-infected cell lysate (Lys) were included as a control. The cytosol and lysate fractions were analyzed directly (lanes 1, 2, and 7) or after immunoprecipitation with anti-rG11 serum (lanes 3 to 6). Anti-rG11 serum did not react with any ^{3}H-labeled proteins in double-shelled (DS) SA11 rotaviruses purified in metrizamide gradients and solubilized in RIPA buffer before immunoprecipitation (lane 6). Molecular weights in thousands are given on the left.

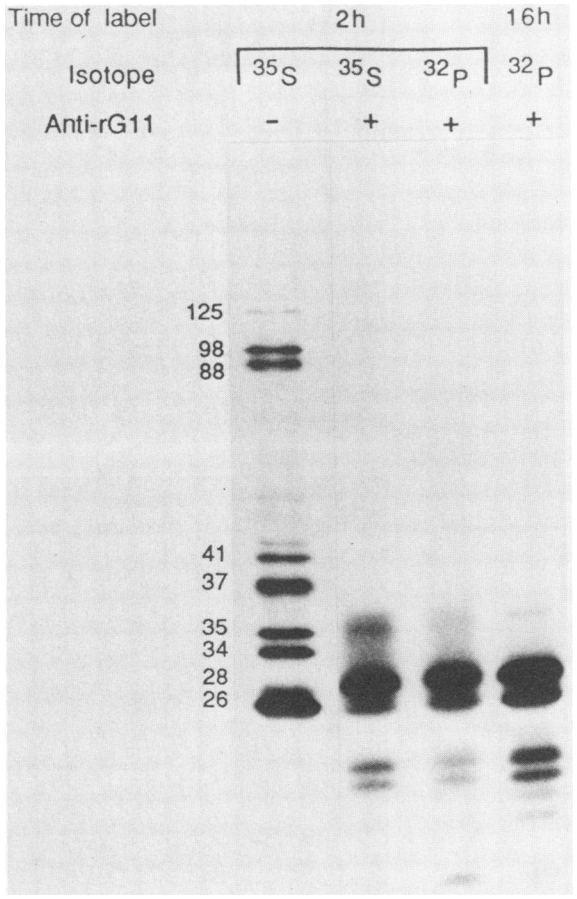

FIG. 8. The gene 11 protein is phosphorylated. Proteins in MA104 cells infected with SA11 were metabolically labeled with $^{32}P_i$. The 28K and 26K bands and 22K and 20K bands were labeled. In addition, some lower-molecular-weight proteins were phosphorylated. Molecular weights in thousands are given on the left.

infected MA104 cells were labeled with $^{32}P_i$, [^{3}H]glucosamine, or [^{3}H]galactose. The 28K and 26K proteins and 22K and 20K proteins were labeled only with phosphate (Fig. 8). Radioactive phosphate was removed from these proteins by treatment with alkaline phosphatase; however, dephosphorylation did not change the electrophoretic migration of these bands (data not shown). Phosphorylation of the 26K protein also occurred in infected insect cells but not in cell-free translation reactions containing [γ-^{32}P]ATP. When infected MA104 cells were labeled with [^{3}H]glucosamine or [^{3}H]galactose, the label was not detectable in cell lysates or immunoprecipitated gene 11 bands. In addition, in vitro labeling of cell lysates with UDP-[^{3}H]galactose and galactosyltransferase (to detect O-linked carbohydrates [15, 34]) was unsuccessful.

DISCUSSION

We expressed the gene 11 protein(s) of simian rotavirus SA11 in *S. frugiperda* cells infected with a baculovirus recombinant (pVL941/SA11-11) containing SA11 gene 11 cDNA. Hyperimmune serum raised against the expressed protein was used to study the gene 11 protein made in SA11-infected MA104 cells. The experimental data in this study confirm our previous finding that the primary gene 11 product has an apparent molecular weight of 26,000 and is modified to a band with an apparent molecular weight of 28,000 (7, 24). Furthermore, this study also detected a minor protein with a molecular weight of 20,000 which was modified to 22,000 in SA11-infected MA104 cell lysates. The 20K protein was also detected in RRL and WG lysate translation systems programmed with SA11 total mRNA. The present study did not determine the exact origin of the lower-molecular-weight bands, but the finding that all four bands are phosphorylated suggests that these bands are all translated from the same ORF. It is possible that the smaller 20K protein is a translation product from the second in-phase AUG present in gene 11. However, the present study cannot exclude the possibility that this is a degradation product or an early termination product of the gene 11 26K primary translation product. The deduced amino acid sequence also contains a potential out-of-phase ORF. The possibility that this ORF is used in cells was not evaluated in this study, but it can now be tested by making and inserting segment 11 cDNA deletion mutants (which lack the first initiation codon) in a transcription vector and then determining whether the protein synthesized in a cell-free translation

system is recognized by anti-rG11 serum. Such experiments are under way (D. B. Mitchell, G. W. Both, S. K. W. Welch, and M. K. Estes, unpublished data).

Previously, we made an antiserum to a denatured 26K protein excised from an SDS-polyacrylamide gel (30). Unfortunately, the low antibody titer of this serum limited its usefulness. Therefore, we used baculovirus-expressed gene 11 protein to generate a high-titer monospecific anti-rG11 serum to characterize the protein product in infected cells and to determine whether it was a minor outer capsid protein or a nonstructural protein. The data presented here show that the gene 11 protein is produced abundantly in virus-infected cells and is not associated with double-shelled virus particles. The deduced amino acid sequences of each gene 11 protein from three rotaviruses show some divergence between amino acid residues 112 and 140, and it has been argued that this variation might reflect antigenic drift of the protein due to its being an outer capsid protein (36). In fact, the major difference among these sequences is due to a frame shift in the sequence of the human Wa rotavirus at residues 123 to 132. Since this protein is nonstructural, the observed variability between the SA11 and UK sequences in this region suggests that this domain is not essential for the functioning of this gene. Several other rotavirus isolates have rearrangements in their gene 11 segments, but these rearrangements do not alter their protein products (26, 34). The cross-reactivity observed for anti-rG11 serum with viruses from all of the known human rotavirus serotypes (including 69M, which contains a rearrangement in its genome segment 11) shows that the gene 11 protein contains conserved epitopes. This broad cross-reactivity and the distinctive staining pattern indicate that this serum could be a useful diagnostic reagent.

This study confirmed earlier results of Ericson et al. (7) which indicated that the gene 11 protein undergoes a post-translational modification that is detectable by a shift in the migration of the band during a chase period. The high serine and threonine residue content of this gene product suggests that modifications of the gene 11 protein could include phosphorylation, O-linked glycosylation, or both. We have demonstrated that the gene 11 protein(s) made in both insect and MA104 cells was labeled metabolically with carrier-free ^{32}P. Similarly, nonstructural phosphoproteins from other viruses in the *Reoviridae* family have been identified (5, 16). However, phosphorylation of our rotavirus protein did not occur in the cell-free translation systems programmed with total mRNA, in contrast to the phosphoproteins of human cytomegalovirus (31) and bluetongue virus (16). An unexpected result was that while each of the different immunoprecipitable gene 11 protein bands incorporated ^{32}P, dephosphorylation of these proteins by treatment with alkaline phosphatase did not change their electrophoretic migration. These results suggest that an additional, still unidentified modification is responsible for the electrophoretic shift of the 26K band to the 28K band. Although attempts to evaluate whether the processing was due to addition of O-linked sugar were unsuccessful, we cannot rule out the possibility that the gene 11 protein contains a few O-linked carbohydrate residues.

Our present studies demonstrated that the gene 11 protein is not associated with virus particles, and therefore we now designate it as a nonstructural protein. An attempt to identify possible functions of this protein also was made by comparing its deduced amino acid sequence with other available sequences in GenBank (Molecular Biology Information Resource, Eugene Software Package, Baylor College of Medicine). The most interesting possible similarity was with several creatine kinases. Our preliminary finding that the gene 11 protein was not phosphorylated in the cell-free translation system suggests that it does not possess kinase activity. However, we cannot exclude the possibility that the in vitro phosphorylation conditions were not optimal; therefore, further work is warranted to determine how the 26K and 28K proteins are phosphorylated. Because phosphorylation of the gene 11 protein occurred in infected monkey kidney cells and in the recombinant virus-infected insect cells, these proteins may be autophosphorylated or they may require other host cell factors. Phosphorylation of other mammalian cell proteins expressed in insect cells has been demonstrated previously (22, 29), and at least in one case, phosphorylation occurred at specific serine and threonine residues, suggesting that insect cells possess a phosphorylation mechanism similar to that of mammalian cells (29).

It is of interest that a phosphorylated nonstructural protein (NS2) of bluetongue virus possesses kinase activity and binds to single-stranded RNA (16). Examination of the gene 11 amino acid sequence also identified the sequence Gly-31-X-X-X-Gly-Arg-Ser-37, which is similar (with Arg-36 being a conservative change from Lys) to the consensus sequence Gly-X-X-X-X-Gly-Lys-Ser (Thr) found in a variety of mononucleotide-binding proteins (12, 19). The presence of this putative mononucleotide-binding sequence in viral proteins suggests that the gene 11 protein is involved in RNA replication. This is consistent with its localization to viroplasms, which are thought to be the sites of RNA replication and assembly of viral cores (30).

On the basis of our data, we conclude that the gene 11 protein is a nonstructural phosphoprotein, and we propose calling the primary product NS26. This conclusion assumes that our hyperimmune serum made to the intracellular protein in insect cells can detect all forms of the protein, including possible altered conformational forms that might be present on virus particles. This study did not address the question of whether the second potential protein product of the gene segment is made. The conclusion that the gene 11 protein is not a structural protein may simplify efforts to develop a subunit vaccine. The availability of a large quantity of monospecific anti-rG11 serum will permit further purification of the gene 11 protein for detailed functional studies, including that of its possible role in the synthesis or assembly of viral RNA in particles.

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