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Use of Recombinant Baculoviruses in Synthesis of Morphologically Distinct Viruslike Particles of Flock House Virus, a Nodavirus

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Flock house virus (FHV) is a small icosahedral insect virus of the family *Nodaviridae*. Its genome consists of two messenger-sense RNA molecules, both of which are encapsidated in the same particle. RNA1 (3.1 kb) encodes proteins required for viral RNA replication; RNA2 (1.4 kb) encodes protein alpha (43 kDa), the precursor of the coat protein. When *Spodoptera frugiperda* cells were infected with a recombinant baculovirus containing a cDNA copy of RNA2, coat protein alpha assembled into viruslike precursor particles (provirions) that matured normally by autocatalytic cleavage of protein alpha into polypeptide chains beta (38 kDa) and gamma (5 kDa). The particles were morphologically indistinguishable from authentic FHV and contained RNA derived from the coat protein message. These results showed that RNA1 was required neither for virion assembly nor for maturation of provirions. Expression of mutants in which Asn-363 at the beta-gamma cleavage site of protein alpha was replaced by either aspartate, threonine, or alanine resulted in assembly of particles that were cleavage defective. For two of the mutants, unusual structural features were observed after preparation for electron microscopy. Particles containing Asp at position 363 were labile and showed a strong tendency to break into half-shells. Particles in which Asn-363 was replaced by Ala displayed a distinct hole in an otherwise complete shell. The third mutant, containing Thr at position 363, was indistinguishable in morphology from authentic FHV.

Flock house virus (FHV) is a nonenveloped icosahedral insect virus of the family *Nodaviridae* (for a review, see reference 15). Its genome consists of two single-stranded, messenger-sense RNA molecules (3, 18), both of which are encapsidated in the same particle (19, 24). RNA1 (3.1 kb) encodes protein A (112 kDa) (3, 8), which is involved in RNA-dependent RNA replication (14, 22). RNA2 (1.4 kb) encodes capsid precursor protein alpha (43 kDa) (1, 2, 8, 10), most of which is cleaved into mature virion coat proteins beta (38 kDa) and gamma (5 kDa). In addition to the genomic RNAs, FHV-infected *Drosophila melanogaster* cells synthesize a subgenomic RNA (RNA3, 0.4 kb), which is derived from RNA1 (11, 13). It encodes protein B (10 kDa) (9), whose precise function is not yet known.

Synthesis of viral progeny in FHV-infected tissue culture cells proceeds via immature nucleocapsids called provirions (12). Provirions are icosahedral particles whose protein shell consists of 180 alpha subunits, presumably arranged with T=3 symmetry (16). The assembly process triggers a proteolytic reaction in the 407-amino-acid alpha chain (12), resulting in cleavage between residues Asn-363 and Ala-364 (16). The cleavage products, proteins beta and gamma, remain part of the mature virion (16). The reaction, which appears to be catalyzed by the coat protein itself (12), occurs deep inside the virus particle near the RNA core (16). The site is inaccessible to proteinase inhibitors and virus-precipitating antibodies (12), and no conditions for blocking maturation are yet known.

Maturation of provirions has at least two functions: it is required for acquisition of virion infectivity (23), and it causes a substantial increase in particle stability (12). The increase in particle stability suggests that the viral shell undergoes structural rearrangements, presumably involving conformational changes at the beta-gamma cleavage site. To gain an understanding of these rearrangements, the three-dimensional structure of virus particles before and after maturation needs to be determined. The structure of the protein shell of mature FHV has already been solved to atomic resolution by X-ray crystallography (5). Analogous studies on the structure of provirions, however, have been hampered by the fact that the spontaneous maturation process cannot be prevented by conventional methods.

We have recently shown that replacement of Asn-363 with Asp, Thr, or Ala results in assembly of cleavage-defective provirions (23). However, these provirions could only be produced by RNA transfection of *Drosophila melanogaster* cells, a procedure unsuitable for generating the large quantities needed for crystallography. To overcome these limitations, we have expressed protein alpha and its cleavage-defective mutants in the more efficient baculovirus expression system, which has been used successfully for synthesis of large amounts of empty particles (reference 25 and references therein). Here, we show that *Spodoptera frugiperda* cells infected with recombinant baculoviruses containing the gene for wild-type protein alpha synthesized high yields of viruslike particles with the same morphology as authentic FHV. Cleavage of protein alpha was normal, suggesting that RNA1 was required neither for capsid assembly nor for maturation. Infection with recombinant viruses carrying mutant alpha protein genes resulted in assembly of particles that were cleavage defective. Two of the mutants,

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sFHV-N363D and sFHV-N363A, showed distinct morphological aberrations, making them potentially valuable tools for elucidation of the FHV assembly pathway. The third mutant, sFHV-N363T, displayed no differences in morphology from authentic FHV and was chosen for crystallographic analysis of the provirion structure (6).

MATERIALS AND METHODS

Cells. *S. frugiperda* cells (line IPLB-Sf21) (26) were propagated at 27°C in TC100 medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 0.35 g of $NaHCO_3$ per liter, 2.6 g of tryptose broth per liter (Difco, Detroit, Mich.), 0.35 g of streptomycin sulfate per liter (Sigma, St. Louis, Mo.), 0.06 g of penicillin-G per liter (Sigma), 0.6 mg of amphotericin B per liter (Sigma), and 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah). Stock cultures were maintained as monolayers in screw-cap plastic flasks (Falcon, no. 3013, 25-cm^2 growth area, and no. 3024, 75-cm^2 growth area). Suspension cultures (200 to 250 ml) were grown in 500-ml spinner flasks (Bellco, Vineland, N.J.).

Infection. Monolayers consisting of 2×10^6 cells per 60-mm-diameter tissue culture plate or 6×10^6 to 10×10^6 cells per 100-mm-diameter tissue culture plate were infected with recombinant *Autographa californica* mononuclear polyhedrosis virus (Ac*M*NPV) at a multiplicity of 5 to 10 PFU per cell. After 1 h at room temperature with rocking, unattached virus was removed by aspiration and cells were covered with growth medium (3 ml per 60-mm plate; 6 ml per 100-mm plate). Plates were further incubated at 27°C without agitation.

Construction of recombinant baculoviruses. A DNA fragment containing the full-length cDNA clone of wild-type FHV RNA2 was released from its cloning vector p2B10SP (4) by digestion with *Hin*dIII and *Bam*HI. cDNA clones of RNA2s containing mutations at the cleavage site of protein alpha were excised 3′ from their respective pBluescript KSII(+) vectors (23) by digestion with the same restriction enzymes. Recessed 3′ termini of the fragments were filled in with Klenow enzyme to generate blunt ends. Transplacement vector pEV55 (17), kindly provided by the Genetics Institute, was digested with *Xho*I, and recessed 3′ termini were filled in with Klenow enzyme. 5′ phosphate residues were removed with calf intestinal alkaline phosphatase, and cDNA fragments of RNA2 were inserted into the vector by blunt-end ligation. To generate recombinant baculoviruses, a monolayer consisting of 2×10^6 *S. frugiperda* cells was rinsed three times with 3 ml of TC100 medium lacking fetal bovine serum and then covered with 3 ml of TC100 medium lacking fetal bovine serum. Transfer vector DNA (15 μg), containing the RNA2 cDNA, was mixed with wild-type L-1 Ac*M*NPV DNA (1 μg) and 30 μg of lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) in a total volume of 100 μl, and the mixture was added to the cells. After incubation for 6 h at 27°C, the medium was removed and replaced with complete growth medium. Incubation at 27°C was continued for 4 days. Progeny virus was plaqued on *S. frugiperda* monolayers by standard procedures (20), and recombinant virus was selected by the occlusion-negative phenotype. Virus stocks were prepared after three to four rounds of plaque purification.

Purification of viruslike particles. Viruslike particles were purified from recombinant Ac*M*NPV-infected *S. frugiperda* cells 4 days after infection. Cell monolayers were dislodged into the growth medium, and the resulting suspension was made 0.5% in Nonidet P-40 and 0.1% in β-mercaptoethanol (2-ME). After incubation on ice for 15 min, cell debris was pelleted in an SS34 rotor at 10,000 rpm for 10 min at 4°C. The supernatant was treated with RNase A (Sigma) at a final concentration of 10 μg/ml at 28°C for 30 min. Viruslike particles were then pelleted through a 1-ml 30% (wt/wt) sucrose cushion in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7)–0.1% 2-ME–0.1% bovine serum albumin (BSA) at 40,000 rpm in an SW41 rotor for 2.5 h at 6°C. The pellet was resuspended in 50 mM HEPES (pH 7)–0.1% 2-ME and layered on a 10-ml 5 to 20% (wt/wt) sucrose gradient in 50 mM HEPES (pH 7)–0.1% 2-ME. Particles were sedimented in an SW41 rotor at 40,000 rpm for 1 h at 11°C. The gradient was fractionated on an ISCO gradient fractionator (model 185) at 0.75 ml/min and 0.5 min per fraction. Fractions containing viruslike particles were pooled and stored at −20°C or further purified on a CsCl gradient. CsCl centrifugation was performed on preformed 10-ml 20 to 45% (wt/wt) gradients in 50 mM HEPES (pH 7)–0.1% 2-ME. Centrifugation was in an SW41 rotor at 36,000 rpm for 16 h at 6°C. The gradient was fractionated as described above. Fractions containing viruslike particles were pooled and diluted fourfold with 50 mM HEPES (pH 7)–0.1% 2-ME. Particles were pelleted in a Beckman TLA centrifuge through a 0.5-ml 30% (wt/wt) sucrose cushion at 100,000 rpm for 20 min at 5°C. The final pellet was resuspended in 50 mM HEPES (pH 7)–0.1% 2-ME and stored at −20°C.

RNA isolation and Northern (RNA) blot analysis. Suspensions of CsCl-purified viruslike particles in 50 mM HEPES (pH 7)–0.1% 2-ME or authentic FHV particles in the same buffer were made 1% (wt/vol) in sodium dodecyl sulfate (SDS) and 0.2 M in NaCl. RNA was extracted twice with an equal volume of phenol-chloroform and precipitated with 2.5 volumes of ethanol at −70°C for 30 min in the presence of 0.25 M sodium acetate, pH 5. The RNA was pelleted, washed with 70% ethanol, dried, and dissolved in nuclease-free water. Aliquots (1 to 2 μg per lane) were electrophoresed through a horizontal 1% agarose gel (10 by 10 by 0.5 cm) in Tris-borate-EDTA at 75 V for 2 h and stained with ethidium bromide. RNA was blotted to Hybond N nylon membrane (Amersham, Arlington Heights, Ill.) by capillary transfer with 10× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) overnight. The membrane was baked at 80°C under a vacuum for 2 h and then prehybridized for 30 min at 60°C in 50 mM sodium phosphate (pH 7.2)–0.8 M NaCl–1 mM EDTA–0.5% SDS–50% formamide–10× Denhardt's reagent (1× Denhardt's reagent contains 0.02% each of Ficoll, polyvinylpyrrolidone, and BSA)–0.25 mg of sheared, denatured salmon sperm DNA per ml. Hybridization was carried out in the same buffer at 60°C overnight.

Radiolabeled RNA probes complementary to virion RNA2 were generated by in vitro transcription from plasmid pSP65 (Promega Biotec, Madison, Wis.) in which a full-length cDNA clone of FHV RNA2 was inserted in antisense orientation downstream of the SP6 RNA polymerase promoter (22). Specifically, transcription reactions were performed in a total volume of 20 μl containing 40 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$; 10 mM NaCl; 2 mM spermidine; 10 mM dithiothreitol; 0.025% BSA; 0.5 mM each of ATP, GTP, and UTP; 0.1 mM CTP; 10 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; Amersham); 40 U of RNasin; 30 U of SP6 RNA polymerase; and 1 to 2 μg of pSP65 linearized with *Hin*dIII. After incubation at 37°C for 2 h, DNA was digested with 2 U of RQ1 DNase (Promega) for 15 min at 37°C and unincorporated nucleotides were removed by filtration through a Sephadex G-50 spin column. Probes with specific activities

TABLE 1. Summary of recombinant baculovirus constructs

Recombinant baculovirus	Gene insert	Amino acids flanking cleavage site between positions 363 and 364 in coat protein alpha
Acαwt	Wild-type alpha protein	Asn-Ala
AcαN363D	Alpha Asn-363→Asp	Asp-Ala
AcαN363T	Alpha Asn-363→Thr	Thr-Ala
AcαN363A	Alpha Asn-363→Ala	Ala-Ala

of about 5×10^6 cpm/μg were used at a final concentration of 0.25 μg/ml during hybridization at 60°C overnight. Blots were washed twice in 2× SSC–0.2% SDS for 15 min at room temperature and once in 0.2× SSC–0.2% SDS at 55 to 60°C for 15 min. The membrane was dried and exposed to Kodak XAR5.

Western blot (immunoblot) analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid)–10% methanol (pH 11) onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Richmond, Calif.) with a Bio-Rad Transblot cell at 50 mA for 10 h. Following transfer, the membrane was incubated in blot buffer (5% [wt/vol] Carnation nonfat dry milk in phosphate-buffered saline [PBS]) for 1 h at room temperature and washed in PBS-Tween buffer (0.05% Tween 20 in PBS) for 5 min. Rabbit anti-FHV serum diluted 1,000-fold in blot buffer was added, and the membrane was gently agitated for 2 h at room temperature and then washed four times in 75 ml of PBS-Tween buffer for 10 min. Incubation was continued for 1 h in blot buffer containing goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugates (Pierce, Rockford, Ill.) at a 5,000-fold dilution. The membrane was washed in PBS-Tween buffer as described above, and antigen-antibody complexes were visualized by incubation in 4-chloro-1-naphthol substrate solution (Pierce) until a purple color developed. Color development was halted by rinsing the membrane with water, and blots were air dried.

Electron microscopy. Purified synthetic FHV (sFHV) particles were applied to carbon-coated copper grids and allowed to absorb for 2 min. Excess solution was removed with filter paper, and the sample was washed twice by floating the grid on droplets of water for 2 min. Particles were then stained with 1% uranyl-acetate and viewed in a Philips EM 420 electron microscope.

RESULTS

Construction of recombinant baculoviruses. Recombinant baculoviruses were constructed according to established protocols (20). In brief, cDNA fragments of FHV RNA2 encoding either wild-type or mutant coat protein alpha were placed under control of the polyhedrin promoter and inserted into the baculovirus genome by homologous recombination. Viruses containing the genes of interest were selected by occlusion-negative phenotype and purified by three to four rounds of plaque purification. All constructs used in this study are summarized in Table 1.

mRNAs of coat protein alpha synthesized in recombinant baculovirus-infected *S. frugiperda* cells were expected to be considerably longer than the 1,400 bases composing authentic FHV RNA2 (Fig. 1). This was because the sequence of RNA2 lacks transcriptional termination and polyadenylation signals which were provided by the flanking polyhedrin sequences downstream of the coat protein gene. We estimated that the final transcripts would be about 2,100 bases long, including a poly(A) tail of 150 bases, which is not present in authentic FHV RNA2.

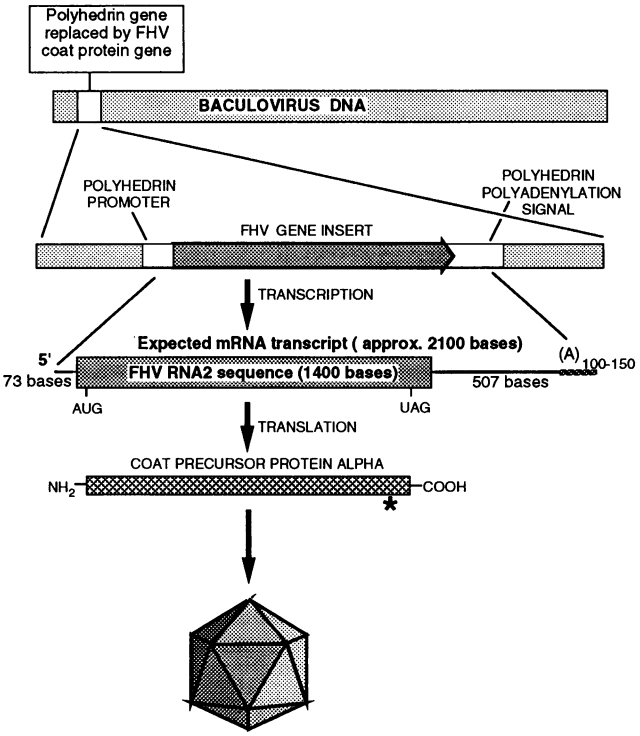

FIG. 1. Construction of synthetic flock house virions (sFHV) with baculovirus recombinants. The first base of the FHV cDNA was positioned 73 bases downstream of the polyhedrin transcriptional start site. RNA transcription proceeds about 500 bases beyond the end of the FHV sequence because of a lack of termination and polyadenylation signals in the coat protein gene. Addition of a poly(A) tail of 100 to 150 bases results in a transcript of about 2,100 bases. The asterisk denotes the approximate location of the beta-gamma cleavage site in coat protein alpha.

The principal question at issue was whether coat protein expressed in the absence of FHV RNA1 would be able to assemble into particles suitable for crystallographic analysis.

Expression of wild-type coat protein alpha in *S. frugiperda* cells. The ability to direct expression of FHV coat protein in *S. frugiperda* cells was first tested for the recombinant containing the wild-type FHV alpha protein gene (Acαwt). Cell extracts from infected cultures were prepared at various times after infection and analyzed on Coomassie brilliant blue-stained SDS-polyacrylamide gels (Fig. 2). Two unique proteins with apparent molecular masses corresponding to those of protein alpha (43 kDa) and its cleavage product beta (38 kDa) were detectable at 48 h. While the level of the 43-kDa protein gradually declined during the course of infection, the 38-kDa protein accumulated in corresponding proportions. This sequence of coat protein synthesis was similar to the pattern observed in FHV-infected *Drosophila melanogaster* cells and strongly suggested that the 43-kDa protein represented capsid precursor alpha whereas the 38-kDa protein represented cleavage product beta. The second cleavage product, gamma (5 kDa), stains poorly with Coomassie brilliant blue (8) and was not visible on the gel. Densitometric analysis showed that the putative alpha and beta proteins constituted about 30% of the total cellular protein on days 3 and 4 after infection.

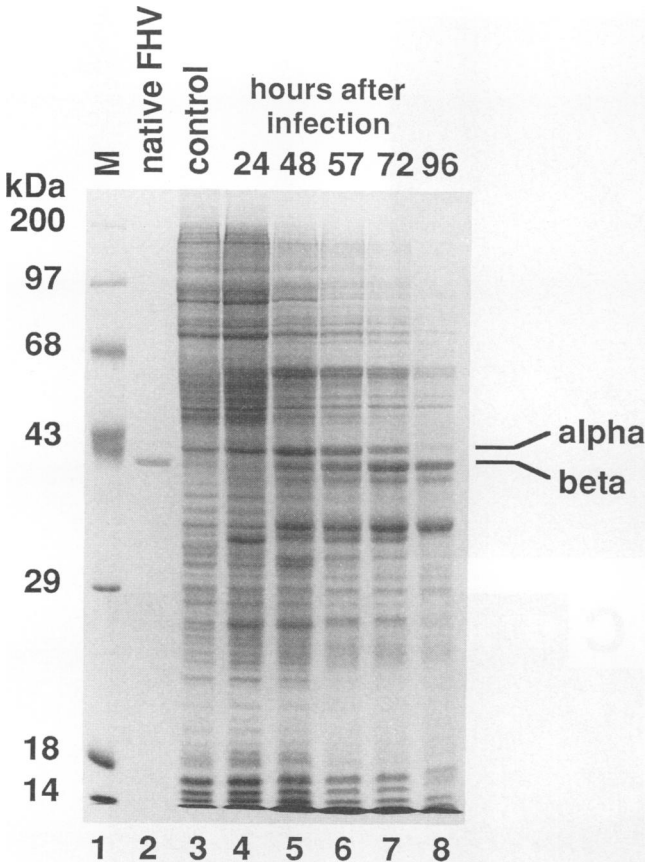

FIG. 2. Time course of protein synthesis in Acαwt-infected *S. frugiperda* cells. Confluent monolayers of *S. frugiperda* cells (2×10^6 cells) were infected with Acαwt at a multiplicity of 20 PFU per cell and incubated at 27°C. At the indicated times, cells were scraped into the plate's medium, collected by centrifugation, and washed twice with 1 ml of PBS. The final pellet was resuspended in 0.1 ml of PBS, diluted with an equal volume of 2× electrophoresis buffer, and heated in a boiling water bath for 10 min. Aliquots corresponding to 2×10^5 cells were fractionated by electrophoresis on an SDS–12% polyacrylamide gel, followed by staining of the gel with Coomassie brilliant blue (23). Lane 1, molecular size markers; lane 2, gradient-purified native FHV; lane 3, mock-infected cell lysate (the protein migrating near protein alpha is a cellular protein, probably actin [11]); lanes 4 to 8, lysates of cells infected with Acαwt.

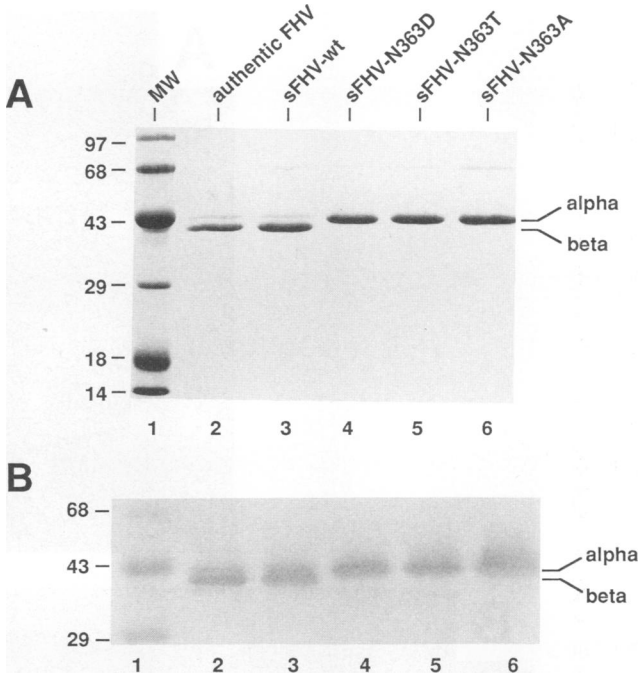

FIG. 3. Electrophoretic analysis (A) and immunodetection (B) of gradient-purified sFHV particles synthesized in recombinant baculovirus-infected *S. frugiperda* cells. (A) sFHV particles were released from infected cells on day 4 after infection and purified by sucrose gradient sedimentation as described in Materials and Methods. Aliquots of 2 μg were fractionated on an SDS–12% polyacrylamide gel, and proteins were visualized with Coomassie brilliant blue. Cleavage product gamma (5 kDa), which stains poorly with Coomassie brilliant blue, is not visible on the gel. (B) Proteins, fractionated by SDS-polyacrylamide gel electrophoresis under conditions identical to those described above, were electroblotted onto polyvinylidene difluoride membrane and incubated with polyclonal anti-FHV serum. Immune complexes were exposed to horseradish peroxidase-conjugated secondary antibodies and visualized as described in Materials and Methods. Lanes 1, molecular weight markers; lanes 2, gradient-purified authentic FHV; lanes 3, sFHV-wt; lanes 4, sFHV-N363D; lanes 5, sFHV-N363T; lanes 6, sFHV-N363A.

Purification of viruslike particles from recombinant baculovirus-infected *S. frugiperda* cells. Cleavage of coat precursor alpha to beta and gamma is dependent on assembly of virions and is not known to occur in the monomeric subunit (12). Detection of protein beta in extracts of Acαwt-infected cultures therefore suggested that cells contained some sort of assembly products, possibly FHV-like particles. To test this hypothesis, putative particles were released from infected cells on day 4 after infection and concentrated by pelleting through a sucrose cushion. Sucrose density gradient sedimentation of the resuspended pellet revealed the presence of material sedimenting at a rate expected for FHV particles (data not shown). Examination of this material by protein gel electrophoresis (Fig. 3A, lane 3) revealed two Coomassie brilliant blue-stained bands, a major and a minor one, which comigrated with the coat proteins of purified authentic FHV (lane 2). Both proteins reacted with FHV antiserum in immunodetection assays (Fig. 3B), confirming their identity as FHV polypeptides. Electron microscopy revealed viruslike particles indistinguishable in morphology from authentic flock house virions (Fig. 4A and B). They were referred to as sFHV-wild type (sFHV-wt). Cosedimentation of [^{35}S]methionine-labeled sFHV-wt with [^{3}H]uridine-labeled authentic FHV on sucrose density gradients showed that synthetic particles sedimented at 130S, i.e., about 5% more slowly than native particles (Fig. 5), suggesting that they contained nucleic acid. This conclusion was further supported by the fact that the ratio of optical density of purified sFHV-wt particles at 260 nm to that at 280 nm was experimentally indistinguishable from that of authentic FHV (Table 2). The yield of sFHV-wt from Acαwt-infected 100-ml cultures (2×10^8 cells) was about 1 to 2 mg (Table 2).

S. frugiperda cells infected with AcαN363D, AcαN363T, or AcαN363A synthesized viruslike particles that were cleavage defective. The mutant particles, referred to as sFHV-N363D, sFHV-N363T, and sFHV-N363A, were similar to sFHV-wt in aspects such as sedimentation coefficient, optical density, and yield (Table 2) but showed no signs of

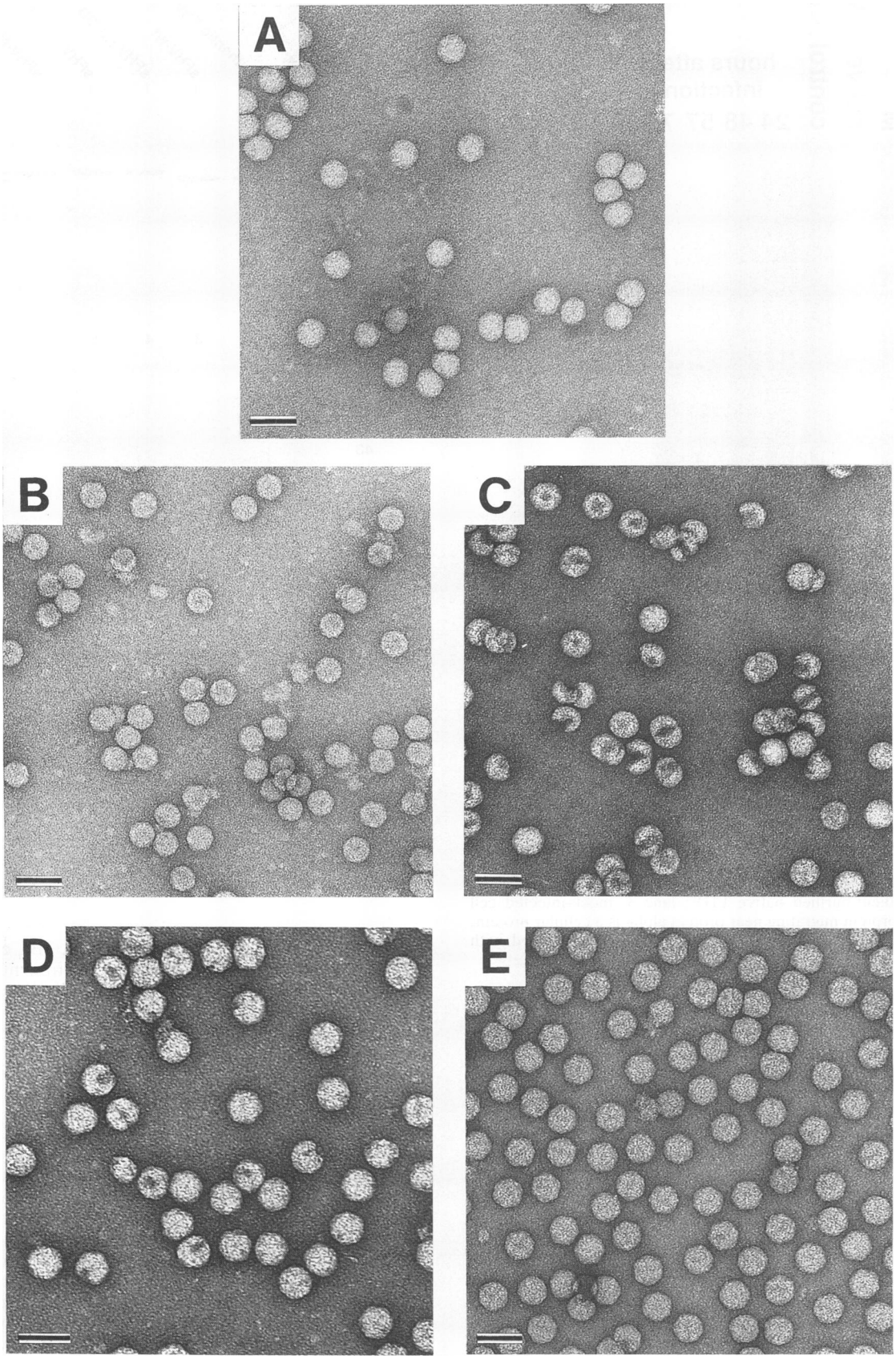
A
B
C
D
E

FIG. 4. Electron micrographs of gradient-purified authentic FHV particles and sFHV particles. Authentic particles were isolated from FHV-infected *Drosophila melanogaster* cells; synthetic particles were isolated from recombinant baculovirus-infected *S. frugiperda* cells. (A) Authentic FHV; (B) sFHV-wt; (C) sFHV-N363D; (D) sFHV-N363A (E) sFHV-N363T. Bar, 50 nm. Note different magnification for panel D (magnification, ×166,000). Magnification for all other panels, ×150,000. The particles in panel E appeared about 10% larger than those in panel B but did not differ in sedimentation rate on the sucrose density gradient (see Discussion).

maturation (Fig. 3, lanes 4 to 6) even after incubation at 26°C for 3 months (data not shown). Thus, they are suitable for crystallographic analysis, which often requires long times for crystal growth.

Electron microscopy revealed a number of unusual structural features for two of the mutants. sFHV-N363D particles (Fig. 4C) were highly heterogeneous and appeared to be rather fragile. This was suggested by the fact that a substantial number of capsids displayed cracks or were split into half-shells. sFHV-N363A particles, on the other hand, contained holes of distinct size in an otherwise complete shell (panel D). sFHV-N363T particles (panel E) showed no distinctive morphological features but were indistinguishable from authentic FHV. This mutant was chosen for crystallographic analysis of the FHV provirion structure (6).

RNA content of viruslike particles. To analyze the nature of nucleic acids encapsidated by synthetic particles, CsCl-purified sFHV-wt was subjected to phenol-chloroform extraction and the resulting material was analyzed by agarose gel electrophoresis (Fig. 6A, lane 2). Instead of the expected band of RNA2, the gel revealed a broad range of RNA sizes with an upper limit of about 4,500 bases, the sum of bases composing RNA1 and -2 of authentic FHV. The extracted nucleic acid was resistant to treatment with DNase but was digested by RNase (data not shown). Identical results were obtained with four independent preparations of sFHV-wt, suggesting that the broad range of RNA lengths observed was not a result of inadvertent degradation of the RNA during purification. In addition, viral RNA extracted in parallel from authentic FHV particles was always intact (Fig. 6A, lanes 3 to 5).

To determine whether the encapsidated population of RNAs contained species derived from the alpha coat protein mRNA, the gel was subjected to Northern blot analysis using a minus-strand RNA2 probe. The broad range of molecular weights (Fig. 6B, lane 2) suggested that most of the hybridized RNA was fragmented, but the intensity of the hybridization signal suggested that a substantial fraction of RNA contained FHV-specific sequences. Moreover, no strong signals were observed for RNA from uninfected control cells (data not shown). To our knowledge, this is the first report that particles expressed from baculoviruses package nucleic acid. However, further studies will be required to determine what fraction of the packaged RNA is actually virus specific.

Two bands in the ethidium bromide-stained agarose gel and on the Northern blot could be distinguished from the remaining background. These bands corresponded in size to RNAs of about 2.1 and 4.0 kb. The 2.1-kb RNA presumably represented the intact alpha protein mRNA, which had terminated at the polyhedrin polyadenylation signal located about 500 bases downstream of the 3′ end of the FHV RNA2 sequence (Fig. 1). However, the polyhedrin gene contains at least two additional polyadenylation signals, 2.1 and 3.6 kb downstream of the first one (7). The 4.0-kb RNA, therefore, might have represented a coat protein transcript that had terminated at the second polyadenylation signal. Further investigations are required to determine whether the encapsidated RNA also included cellular or Ac*M*NPV RNA.

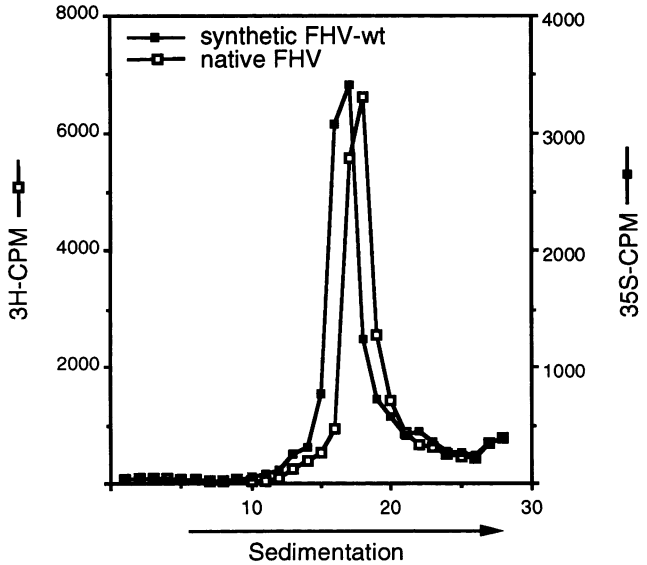

FIG. 5. Sedimentation profile of sFHV-wt isolated from Acαwt-infected *S. frugiperda* cells. [^{35}S]methionine-labeled sFHV-wt (17,000 cpm) in 50 mM HEPES (pH 7)–0.1% 2-ME was mixed with [^{3}H]uridine-labeled native FHV (19,000 cpm) and centrifuged through a 5-ml gradient of 5 to 20% (wt/wt) sucrose in the same buffer. Centrifugation was in a Beckman SW50.1 rotor at 45,000 rpm for 30 min at 20°C. The gradient was fractionated on an ISCO model 185 fractionator and counted after adding 200 μl of water and 5 ml of scintillation fluid (Ecoscint) to each fraction.

DISCUSSION

We have shown that viruslike particles of FHV (sFHV) could be expressed from recombinant baculoviruses in yields of 1 to 2 mg of particles per 100 ml of cultured cells, sufficient for crystallographic analysis of structural changes involved during maturation cleavage of the nodaviral shell.

TABLE 2. Physicochemical properties of sFHV particles isolated from recombinant baculovirus-infected *S. frugiperda* cells

Synthetic particles	Yield (mg)[a]	$OD_{260/280}$[b]	$s_{20,w}$[c]	Cleavage of alpha protein
sFHV-wt	1–2	1.56	130	Yes
sFHV-N363D	1–2	1.64	130	No
sFHV-N363T	1–2	1.59	130	No
sFHV-N363A	1–2	1.53	130	No

[a] From 2 × 10^8 infected *S. frugiperda* cells.

[b] Ratio of optical density (OD) at 260 nm to that at 280 nm; the ratio of optical density of gradient-purified authentic FHV at 260 nm to that at 280 nm is 1.56.

[c] Determined by cosedimentation of [^{35}S]methionine-labeled synthetic particles and [^{3}H]uridine-labeled authentic particles on isokinetic 5 to 20% (wt/wt) sucrose gradients.

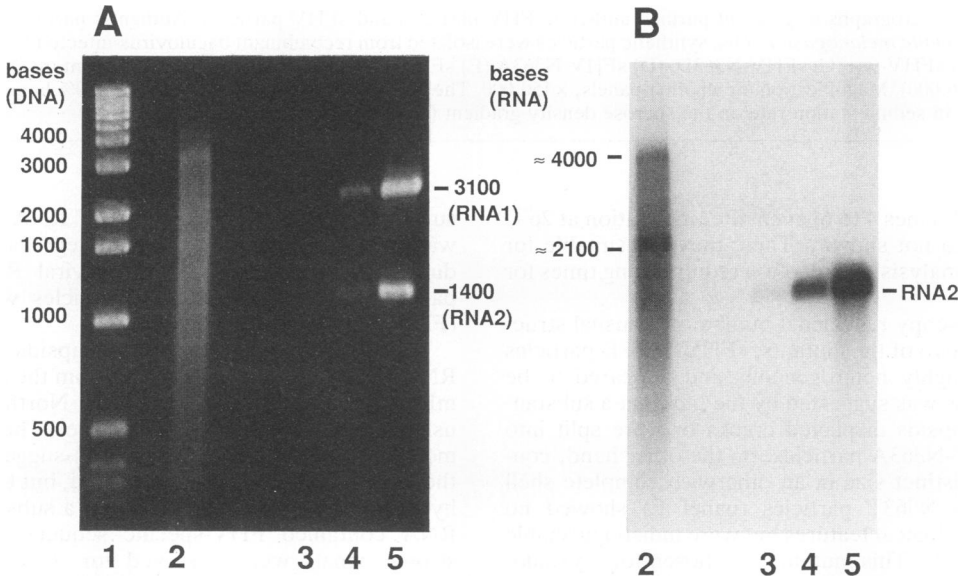

FIG. 6. Electrophoretic comparison of RNA encapsidated by sFHV-wt and authentic FHV. (A) RNA was phenol-chloroform extracted from CsCl-purified sFHV-wt and sucrose density gradient-purified authentic FHV, fractionated on a 1% agarose gel, and stained with ethidium bromide. Lane 1, 1-kb DNA ladder; lane 2, RNA (1.6 μg) extracted from sFHV-wt; lanes 3 to 5, RNA extracted from authentic FHV (0.01, 0.1, and 1 μg, respectively). (B) Northern blot analysis of the same gel probed with an in vitro-synthesized, ^{32}P-labeled, minus-strand RNA2 transcript. Lanes are the same as for panel A.

These studies also provide the first evidence that RNA1, which is required for replication of the FHV genome (11), is dispensable for virion assembly.

sFHV-wt particles were similar in morphology and cleavage behavior to authentic FHV but sedimented 5% more slowly on sucrose density gradients. The slower sedimentation velocity might be due to a slightly larger diameter in sFHV particles or to encapsidation of a subnormal amount of RNA. Electron microscopy revealed no obvious differences in the diameters of authentic and synthetic particles, but a 5% increase taxes the limits of detection by this method. Arguing against a subnormal complement of RNA are the ratios of optical densities of synthetic and authentic particles at 260 nm to those at 280 nm (Table 2) and the narrow band widths on sucrose density gradients (Fig. 5). RNA extracted from sFHV-wt was highly heterodisperse, ranging in size from several hundred to about 4,500 bases (Fig. 6), and a much wider range of sedimentation velocities might have been expected had the amount of RNA packaged in each particle differed markedly.

One of the most striking results of our studies was the marked morphological differences among the three cleavage-defective mutants. The N363T mutants (Fig. 4E) were morphologically indistinguishable from particles containing wild-type protein (Fig. 4A and B). However, a substantial fraction of the N363D mutants were split into half-shells (Fig. 4C), and the N363A mutants displayed a discrete hole (Fig. 4D). This difference indicates that the region occupied by Asn-363 is a critical element for the overall particle stability. Because all four of the sFHV particles cosedimented in solution, it is likely that they were properly assembled but acquired different morphologies by dissociation along distinctive pathways during preparation of the particles for electron microscopy. The largely intact degradation products suggest that the altered residues (Asp and Ala) add strain to the assembled subunits that is relieved in different ways for each mutation. If such dissociation pathways could be duplicated in solution, characterization of the subparticles produced might provide important insights into the assembly and dissociation of nodaviruses, much as the 5S and 14S subparticles provided a Rosetta stone for deducing the dissociation and assembly pathway of picornaviruses (21).

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