Reconstitution of Flock House Provirions: a Model System for Studying Structure and Assembly

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Assembly of Flock House virus in infected *Drosophila* cells proceeds through an intermediate, the provirion, which lacks infectivity until the coat precursor protein, alpha, undergoes a spontaneous “maturation” cleavage (A. Schneemann, W. Zhong, T. M. Gallagher, and R. R. Rueckert, *J. Virol.* 66:6728, 1992). We describe here methods for purifying provirions in a state which permitted dissociation and reassembly. Dissociation, to monomeric alpha protein and free RNA, was accomplished by freezing at pH 9.0 in the presence of 0.5 M salt and 0.1 M urea. When dialyzed at low ionic strength and pH 6.5, the dissociation products reassembled spontaneously to form homogeneous provirions with a normal complement of RNA as judged by cosedimentation with authentic virions and by ability to undergo maturation cleavage with acquisition of substantial, though subnormal, infectivity. Reconstitution experiments, i.e., remixing components after separating RNA from capsid protein, generated abnormal particles, suggesting the presence in the unfractionated dissociation products of an unidentified “nucleating” component.

Flock House virus (FHV) is a nonenveloped, icosahedral insect virus of the family *Nodaviridae* (for a review, see reference 15). Its small genome consists of two messenger-sense RNA molecules (4, 5, 7), both of which are thought to be encapsidated in a single virion (19, 20, 26). RNA1 (3.1 kb) encodes protein A (112 kDa) (4, 5), the putative RNA-dependent RNA polymerase (14, 21); RNA2 (1.4 kb) encodes protein alpha (43 kDa) (2, 3, 7, 9), the precursor of the coat protein. A nonencapsidated subgenomic RNA derived from RNA1 (11, 13) encodes a third protein, protein B (10 kDa) (8), whose precise function is not yet known.

Studies on FHV assembly in cultured *Drosophila* cells have shown that virions are first synthesized as labile precursor particles called provirions (12). The provirion capsid is composed of 180 alpha subunits arranged with T=3 symmetry (16). Following assembly of the viral shell, alpha subunits cleave spontaneously into mature coat proteins beta (38 kDa) and gamma (5 kDa) (12). The maturation reaction is slow, with half of the alpha subunits being cleaved in 4 h at room temperature or in 2 days at 4°C (12). This slow cleavage rate contrasts sharply with the rapidity of provirion assembly, which occurs within minutes after alpha protein synthesis (12). The cleavage reaction results in increased particle stability (12) and is required for acquisition of virion infectivity (23).

X-ray crystallographic analysis of mature FHV particles has shown that the 180 protein subunits are clustered into 60 triangular units also known as asymmetric units (6). At the center of each asymmetric unit, three protein chains are twisted together via prominent peptide loops. The characteristic geometry of each virion is the result of a precise pattern of alternating flat and bent contacts between the 60 asymmetric units. The two modes of interaction are due to slightly different

conformations of the chemically identical protein chains. At the flat contacts an internal segment of protein folds into the joint between the interacting subunits, preventing them from forming the dihedral angle that is observed at the bent contacts. In addition, the flat joints are stabilized by an ordered segment of genomic RNA (6).

As a tool to study the pathway of nodavirus assembly, including the mechanism that ensures encapsidation of two unrelated RNA molecules into the same particle, we have developed an *in vitro* assembly system that allows reconstitution of virus particles under controlled experimental conditions. The first requirement for the development of such a system was to establish a practical source of coat protein subunits. Provirions were chosen for this purpose because, in contrast to mature virions, they contain coat protein in its uncleaved precursor state, i.e., the protein subunit through which assembly proceeds *in vivo*. Here we show that provirions could be quantitatively dissociated into monomeric alpha protein and free viral RNA by freezing at pH 9 and moderate ionic strength in the presence of EDTA. Mature virions remained completely stable and did not dissociate under the same conditions. When dissociated provirions were dialyzed at pH 6.5 and low ionic strength, they reassembled to form virus particles that cosedimented with authentic virions on density gradients. Alpha chains cleaved normally into beta and gamma chains, and the particles showed substantial infectivity. In contrast, reconstitution of FHV after separation of its protein and RNA components yielded abnormal particles that sedimented slightly more slowly than authentic virions and that showed little, if any, infectivity. We propose that a “nucleating” structure is essential for accurate formation of Flock House virions and present a preliminary model for the pathway of nodavirus assembly.

MATERIALS AND METHODS

Propagation of FHV. *Drosophila* cells (Schneider's line 1) (25) were suspended to 4×10^7 cells per ml in Schneider's insect medium (24) supplemented with 15% fetal bovine serum (complete growth medium). Gradient-purified FHV (12) was

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added to a multiplicity of 120 PFU per cell and allowed to attach for 1 h at 26°C with occasional mild agitation. Cells were then sedimented and resuspended to 5×10^6 cells per ml in complete growth medium, and 20-ml aliquots were distributed into 100-mm-diameter tissue culture plates. If RNA-labeled virus was to be prepared, [^3H]uridine (5 $\mu\text{Ci/ml}$; DuPont, Wilmington, Del.) was added at this point. To prepare virus labeled in the coat protein, medium was removed at 15 h postinfection, and the cell monolayers were rinsed once with 10 ml of PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] buffer (25 mM PIPES [pH 6.8], 100 mM NaCl, 0.1% bovine serum albumin [BSA]). Methionine-deficient Grace's insect medium (GIBCO BRL, Gaithersburg, Md.), supplemented with 50 to 100 μCi of [^{35}S]methionine (Amersham, Arlington Heights, Ill.) per ml, was added to the culture dishes, and plates were incubated for another hour at 26°C before isolation of provirions.

The intervals chosen for radiolabeling distinguished virions assembled early in the infectious cycle ([^3H]uridine labeled) from those synthesized during the hour before harvesting ([^3H]uridine and [^{35}S]methionine labeled).

Purification of provirions. Provirions were isolated as described elsewhere (23). Briefly, FHV-infected cells were lysed with detergent at 16 h postinfection, and virus in the supernatant was pelleted through linear 10 to 30% sucrose gradients. Pellets were resuspended in 225 μl of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7) and pooled. A 25- to 50- μl aliquot was removed for determination of optical density (with 4.15 units of optical density at 260 nm corresponding to 1 mg/ml) and specific radioactivity. Yields of 0.8 to 1.0 mg were typically obtained from a single 100-mm-diameter tissue culture dish containing 10^8 cells.

Dissociation of provirions. Immediately after resuspension of freshly isolated provirions in 5 mM HEPES (pH 7), an equal volume of $2\times$ standard dissociation buffer (D-buffer) (0.2 M Tris-HCl [pH 9], 0.2 M urea, 1.0 M NaCl, 20 mM EDTA, 10 mM dithiothreitol [DTT], 0.4 mM phenylmethylsulfonyl fluoride [PMSF], 0.4 mM tosyl-lysylchloro ketone [TLCK], 2 μg of human α_2 -macroglobulin per ml, 1 U of Inhibit-ACE [5 Prime $\rightarrow$ 3 Prime Inc., Boulder, Colo.] per 30 μl) was added to the sample, and the mixture was incubated at -20°C for at least 3 h. D-buffer was used in all provirion dissociation experiments except those for which the conditions are specifically described in Results. Proteinase inhibitors and RNase inhibitors were present in all cases.

Gel filtration chromatography. Gel filtration was performed on a Sephacryl S-200 column (1.5 by 46 cm; Pharmacia Biotech Inc., Piscataway, N.J.). The gel matrix, provided in suspended form by the manufacturer, was autoclaved to inactivate RNases and equilibrated in D-buffer lacking proteinase and RNase inhibitors. Dissociated virus (0.3 to 0.8 ml) was applied to the column and eluted at 17 ml/h. Fractions (0.5 ml) were collected, and the radioactivity in each fraction was determined by liquid scintillation counting.

Reconstitution of dissociated provirions. Provirions (200 μl), dissociated in D-buffer, were reconstituted by dialysis against 200 ml of assembly buffer {0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 6.5), 0.1 M NaCl, 5 mM CaCl_2 , 5 mM MgCl_2 , 0.1% 2-mercaptoethanol (2-ME), 0.2 mM PMSF} at room temperature for at least 4 h but usually overnight. The products were combined with [^3H]uridine-labeled authentic FHV and analyzed on 5-ml linear 5 to 20% (wt/wt) sucrose gradients in assembly buffer. Centrifugation was in a Beckman SW50.1 rotor at 45,000 rpm for 30 min at 20°C . Gradients were fractionated with an ISCO model 185 at 0.375 ml/min and 0.5 min per fraction. The radioactivity in

each fraction was determined by liquid scintillation counting after addition of 0.2 ml of water and 5 ml of scintillation cocktail (Ecoscint) to each fraction.

Purification of protein alpha. [^{35}S]methionine-labeled provirions (1 ml), dissociated in D-buffer for 24 h at -20°C , were layered on top of a linear 11-ml 5 to 20% (wt/wt) sucrose gradient in D-buffer lacking proteinase inhibitors and RNase inhibitors. The gradient was centrifuged in a Beckman SW41 rotor at 40,000 rpm for 6 to 10 h at 5°C and fractionated with an ISCO model 185 at 0.75 ml/min and 0.5 min per fraction. Alpha protein was located by determination of the radioactivity in an aliquot (8 μl) of each fraction by liquid scintillation counting. If protein was not radiolabeled, fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis.

In vitro assembly of coat protein alpha. [^{35}S]methionine-labeled protein alpha (200 μl) at a concentration of 0.1 to 0.2 mg/ml was mixed with FHV genomic RNA at a protein/RNA ratio of 5:1 (wt/wt). RNase inhibitor (Inhibit-ACE) was added (1 U/30- μl reaction volume), and the mixture was dialyzed overnight against 200 ml of assembly buffer at room temperature. The sample was analyzed by sedimentation on a linear 5-ml 5 to 20% (wt/wt) sucrose gradient in assembly buffer in the presence of [^3H]uridine-labeled authentic FHV. Centrifugation was in a Beckman SW50.1 rotor at 45,000 rpm for 30 min at 20°C . The gradient was fractionated with an ISCO model 185 (0.375 ml/min; 0.5 min per fraction), and the radioactivity in each fraction was determined by liquid scintillation counting.

Electrophoretic analysis of [^{35}S]methionine-labeled proteins. Electrophoresis was performed on discontinuous SDS-polyacrylamide gels according to the procedure of Laemmli (18). Samples were mixed with an equal volume of $2\times$ electrophoresis buffer (125 mM Tris-HCl [pH 6.8], 4.6% SDS, 10% 2-ME, 20% glycerol) and heated in a boiling water bath for 5 min. Slab gels (14 cm by 16 cm by 0.75 cm) contained 12% (wt/vol) acrylamide in the resolving gel (14-cm length) and 5% (wt/vol) acrylamide in the stacking gel (1-cm length). Electrophoresis was at 150 V for 6 h. Gels were fixed, dried, and exposed to Kodak XAR-5 film.

RESULTS

Quantitative dissociation of provirions without dissociation of mature virions. Purified provirions were dissociated by freezing the preparation overnight at -20°C in a specially developed pH 9 dissociation buffer (D-buffer) consisting of 0.5 M NaCl, 0.1 M urea, 0.1 M Tris-HCl (pH 9), 10 mM EDTA, and 5 mM DTT with selected proteinase and RNase inhibitors. The integrity of the provirion preparation before dissociation and the identity of its sedimentation pattern with that of mature virions are shown in Fig. 1A. As seen in Fig. 1B, dissociation was specific for provirions; mature virions included as a control survived completely intact. The slightly slower sedimentation of mature virions in the gradient shown in Fig. 1B was due to the greater density of high-salt D-buffer. Use of D-buffer in the gradient was necessary to prevent partial reaggregation of dissociated material which occurred with the neutral buffer employed for Fig. 1A. Time course experiments revealed that dissociation of provirions in D-buffer was complete within 30 min after the sample (typically 0.5 to 1.0 ml) was placed in a 1-dram (ca. 4-ml) glass vial in a freezer at -20°C (data not shown).

Key variables affecting dissociation of provirions. The use of EDTA during the purification of provirions was of key importance for preparing dissociable particles. This included wash-

FIG. 1. Sedimentation of FHV provirions before (A) and after (B) the dissociation procedure. [^{35}S]methionine-labeled provirions (60,000 cpm) were resuspended in 5 mM HEPES (pH 7) and mixed with mature virions differentially labeled with [^3H]uridine (60,000 cpm); the mixture of differentially labeled particles was divided into equal aliquots and brought to the buffer conditions indicated in each panel by adding an equal volume of $2\times$ buffer. Samples (100 μL) were layered on linear 5-ml gradients of 5 to 20% (wt/wt) sucrose in the indicated buffers. The sample shown in panel A was centrifuged immediately after preparation; the other one (B) was centrifuged after 24 h at -20°C . Centrifugation was in a Beckman SW50.1 rotor at 45,000 rpm for 30 min at 20°C . Each gradient was fractionated on an ISCO model 185, and the radioactivity in each fraction was determined by liquid scintillation counting.

ing infected cells with EDTA before releasing virus with detergent to avoid exposure of provirions to divalent cations present in the culture medium. Provirions purified in the absence of EDTA were almost completely refractory to dissociation even though EDTA was included later in D-buffer (data not shown).

Other variables affecting the degree of provirion dissociation included storage temperature and urea. Provirions stored at 0 or -75°C were less efficiently dissociated than those stored at -20°C (Table 1). Dissociation was also incomplete when urea was omitted from D-buffer, although the effect was not as pronounced at -20°C as it was at -75 and 0°C ; in the absence of urea, 14% of the provirions survived at -20°C , while 47 and 48% survived incubation at -75 and 0°C , respectively (Table 1).

The effect of pH and ionic strength on dissociability of

TABLE 1. Effect of temperature and urea on dissociation of provirions^a

Temp ($^\circ\text{C}$)	D-buffer	% Radioactivity remaining in 137S particles
0	Complete	13
	Minus urea	48 ^b
-20	Complete	<5 ^c
	Minus urea	14
-75	Complete	20
	Minus urea	47

^a Provirions, purified as described in Materials and Methods, were stored for 24 h at the indicated temperatures in D-buffer with or without 0.1 M urea. Samples were then analyzed on sucrose density gradients containing D-buffer modified as indicated.

^b Incubated in D-buffer for 8 h only.

^c Background level; no peak was apparent.

provirions in the absence of urea is shown in Table 2. At 0.5 M NaCl, high efficiency of dissociation was observed at alkaline pH but not at neutral or slightly acidic pH. Thus, at pH 8 and 9 only 14% of the radioactivity was associated with the 137S provirion peak, whereas at pH 6 and 7, 65 to 75% of the particles remained intact (Table 2). At 1 M NaCl, however, provirions also dissociated efficiently at pH 7. When the salt concentration was increased to 2 or 2.5 M NaCl, the extent of dissociation decreased and provirions disrupted into subparticles, which sedimented at 50 to 60S. These subparticles were also observed when provirions were dissociated in D-buffer lacking NaCl entirely (data not shown).

Although provirions incubated in D-buffer appeared to be completely dissociated as judged by gradient sedimentation profiles (Fig. 1B), only 60 to 70% of the radioactivity applied to the gradient was recovered. Treatment of dissociated provirions with SDS at a final concentration of 1% (wt/vol) increased recovery to 90%, suggesting that in the absence of detergent a fraction of the coat protein pelleted during centrifugation (data not shown). Systematic modifications of the dissociation conditions, including temperature, ionic strength, pH, and urea concentration, did not lead to improved recovery of soluble coat protein.

Evidence for mature virions in provirion preparations.

Synthesis of coat protein alpha in FHV-infected *Drosophila* cells reaches maximum levels between 16 and 20 h postinfection at 26°C . The newly synthesized subunits assemble within minutes into provirions whose genomic RNA is derived from a pool of unpackaged RNAs 1 and 2 that accumulate in the cell during infection (12). A sedimentation profile of dissociated

TABLE 2. Effect of pH on dissociation of provirions^a

pH	NaCl concn (M)	% Radioactivity remaining in 137S particles
6	0.5	66
	1.0	52 ^b
7	0.5	72
	1.0	5
8	0.5	14
	1.0	8
9	0.5	14
	1.0	9

^a Provirions, purified as described in Materials and Methods, were stored for 24 h in D-buffer at the indicated pH and NaCl concentration. Urea was not present. Samples were analyzed on sucrose density gradients in the respective D-buffer.

^b Material sedimenting as a broad 60 to 120S peak.

FIG. 2. Evidence that provirions contain minor amounts of mature virions, which are not evident in the [^{35}S]methionine-labeled preparation. [^3H]uridine (100 μCi) was added to a monolayer of *Drosophila* cells (10^8 cells) from 1 to 15 h after infection. At 15 h, the time of peak coat protein synthesis, the medium was replaced with 5 ml of fresh medium containing [^{35}S]methionine (250 μCi). Provirions were purified at 16 h and dissociated in D-buffer. A sample (100 μl) was layered on a 5-ml 5 to 20% (wt/wt) sucrose gradient in D-buffer lacking proteinase inhibitors and RNase inhibitors; centrifugation was for 30 min at 45,000 rpm in an SW50.1 rotor at 20°C.

provirions labeled with [^3H]uridine from 1 to 15 h and with [^{35}S]methionine from 15 to 16 h is shown in Fig. 2. The bulk of viral RNA was liberated during incubation in D-buffer, but a small fraction, representing about 14% of the total ^3H counts per minute, remained associated with 137S particles. That these particles represented mature or partially matured virions was shown by gel electrophoresis, which confirmed the presence of proteins alpha, beta, and gamma in the 137S peak, whereas only protein alpha was found in the top fractions of the gradient (data not shown). In the same preparation, provirions radiolabeled from 15 to 16 h with [^{35}S]methionine showed no evidence of undissociable particles. This is because the time required for maturation cleavage ($t_{1/2} = 4$ h at the growth temperature) was long relative to the labeling period, so that newly formed provirions had not yet had time to undergo much cleavage before harvest and purification.

Characterization of provirion dissociation products. A considerable body of evidence supports the idea that each nodavirus contains one molecule each of RNAs 1 and 2, but direct proof is still lacking since the usual methods of isolating the RNA from virions or infected cells yield the RNA molecules in dissociated form. If nodavirus RNAs 1 and 2 were linked through 16 bp (4), then the paired molecules should survive intact (17) under the gentle high-salt conditions here used to dissociate provirions. Such a 1:1 complex might be expected to sediment at about 29S (see Discussion). However, RNA liberated with D-buffer sedimented as two peaks (Fig. 3) which were shown by electrophoresis on agarose gels to correspond to RNAs 1 and 2, respectively (not shown). About 20% of the material was heterodisperse, sedimenting ahead of RNA1, but revealed no hint of a 29S peak. The dissociated RNA strands produced by D-buffer therefore provided no new insights into the nature of the mechanism by which the genomic molecules might be selected for packaging.

FIG. 3. Gradient sedimentation profile showing RNA products of provirion dissociation. Preparation of provirions was as described in the legend to Fig. 2 except that actinomycin D (5 $\mu\text{g}/\text{ml}$) was added at 10 h postinfection to inhibit cellular RNA synthesis and that [^3H]uridine was present from 10.5 to 15 h. After dissociation of provirions in D-buffer, a sample (250 μl) was centrifuged through a 12-ml 5 to 20% (wt/wt) sucrose density gradient in D-buffer for 6 h at 40,000 rpm in an SW41 rotor at 5°C.

Evidence that provirion subunits dissociated to the monomeric state. The oligomeric state of the protein subunits released by freeze-thawing provirions with D-buffer was examined by gel filtration chromatography using a matrix of Sephacryl S-200. With a nominal fractionation range of 5 to 250 kDa, this gel should exclude viral RNA, virions, and any oligomer of coat protein exceeding five molecules of alpha subunits. Figure 4A shows a typical elution profile with a provirion preparation in which the differentially labeled RNA and protein were well separated. Virtually all of the RNA label eluted in the void volume (fraction 20), while most of the coat protein eluted as a homogenous peak at fraction 49. The apparent molecular weight of protein alpha in this peak was determined by gel filtration in the presence of differentially labeled molecular weight markers (Fig. 4B). The majority of the protein eluted in the vicinity of ovalbumin (molecular weight, 45,000). The calibration curve constructed from elution volumes of the standard proteins (data not shown) indicated a mass of 41 kDa for the alpha protein, close to the theoretical value of 43 kDa predicted by its nucleotide sequence (2). Thus, provirion shells dissociated all the way to the monomeric subunit.

A small amount of coat protein consistently eluted with RNA and undissociated virions in the void volume. In four experiments, the proportion varied from 7 to 25% of the total protein, corresponding to 13 to 45 alpha subunits per virion. That the protein peak in the void volume was not entirely due to intact virus particles was suggested by the observation that the 137S region of the gradient shown in Fig. 2 contained only background levels of protein. To test for aggregates like those detected during ultracentrifugation of dissociated provirions, the material eluting in the void volume was sedimented on a sucrose gradient in D-buffer. Of the applied [^{35}S]methionine label, 80% pelleted, while the remaining 20% formed a broad

FIG. 4. Gel filtration profile of dissociated provirions on a Sephacryl S-200 column. (A) [^3H]uridine- and [^{35}S]methionine-labeled provirions (0.4 ml), dissociated in D-buffer, were applied to the column and eluted with D-buffer lacking proteinase inhibitors and RNase inhibitors as described in Materials and Methods. The radioactivity in each fraction (0.5 ml) was determined by liquid scintillation counting. (B) [^{35}S]methionine-labeled provirions (0.25 ml) were dissociated in D-buffer and mixed with four ^3H -labeled protein standards (MW markers): aldolase (161 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (30 kDa). The mixture was applied to the column and eluted as described for panel A.

peak halfway between viral RNA and undissociated particles (data not shown). Thus, the majority of the protein in the void volume presumably represented aggregates, which were not further characterized.

Reassembly of dissociated provirions. As already noted, dissociated coat protein had a marked tendency to reaggregate at neutral pH. Preliminary trials revealed that dissociated provirions could be reassembled by simply dialyzing the mixture against 0.1 M MES (pH 6.5)–0.1 M NaCl–5 mM MgCl_2 –5 mM CaCl_2 –0.1% 2-ME–2 mM PMSF (assembly buffer). As seen in Fig. 5, reassembly seemed to be nearly quantitative, and the products cosedimented with mature virions. Most of the coat protein (typically about 80%) that was present as slowly sedimenting material after dissociation could be recovered in the 137S peak after reassembly; protein that was present in aggregated form was lost in the pellet. Assembly was already complete within 3 h, the shortest dialysis interval tested. Electrophoretic analysis of RNA extracted from reassembled provirions revealed that both RNAs 1 and 2 were present in the same proportion as in virions (data not shown).

Addition of divalent cations to the assembly buffer was not required for reassembly. However, in the presence of 10 mM EDTA, assembly was almost completely inhibited (data not shown). This behavior may reflect trace contamination by divalent cations in one or more of the buffer components.

FIG. 5. Sedimentation analysis of authentic and reassembled FHV particles on a sucrose density gradient. Dissociated [^{35}S]methionine-labeled provirions (200 μl) were dialyzed at room temperature against assembly buffer (0.1 M MES [pH 6.5], 0.1 M NaCl, 5 mM MgCl_2 , 5 mM CaCl_2 , 0.1% 2-ME, and 2 mM PMSF). Differentially labeled mature virions were then added, and the mixture was centrifuged through a linear 5-ml gradient of 5 to 20% (wt/wt) sucrose in assembly buffer. Centrifugation was in an SW50.1 rotor at 45,000 rpm for 30 min at 20°C. The gradient was fractionated, and the radioactivity in each fraction was determined by liquid scintillation counting. The stippled box at fraction 30 (P) indicates that some radioactivity (typically 25 to 40%) pelleted during centrifugation; see text for details.

Reassembly was also inhibited if dissociated provirions were treated with RNase before dialysis, suggesting that RNA played an important role in assembly of virus particles (data not shown).

Reassembled provirions undergo maturation cleavage. The extent of alpha protein cleavage in reassembled particles was determined by SDS-polyacrylamide gel electrophoresis, as shown in Fig. 6. Lane 1 contains [^{35}S]methionine-labeled virus taken from the provirion preparation used for dissociation and reassembly. The particles were incubated for 24 h at room temperature to show the migration of proteins alpha, beta, and gamma. The band representing protein gamma is diffuse and usually is hard to detect because the gamma chain is small (44 amino acids) and contains only one methionine residue. Gamma chains migrated ahead of the dye front under our electrophoresis conditions.

As seen in Fig. 6, lane 2, provirions from the same preparation, dissociated immediately after purification, showed no cleavage products. Lane 3 of Fig. 6 contains virions reassembled at pH 6.5 for 24 h by using the dissociated material shown in lane 2. Most of the protein cleaved into beta and gamma chains, with the extent of cleavage being comparable to that of the control sample (lane 1). The thin band located just behind gamma and present in all three lanes is an artifact created by the dye front.

Infectivity of reassembled particles. The infectivity of reassembled FHV particles was determined by plaque assay on *Drosophila* cells, using samples analogous to those described

FIG. 6. Autoradiogram showing cleavage of coat precursor protein alpha in reassembled FHV particles. Purified [^{35}S]methionine-labeled provirions in 5 mM HEPES (pH 7) were divided into two aliquots. One aliquot was diluted fourfold into 50 mM HEPES (pH 7)–0.1% 2-ME and incubated at room temperature. After 24 h, the sample was mixed with an equal volume of 2 $\times$ electrophoresis buffer, heated in a boiling water bath for 10 min, and stored frozen until analysis (lane 1). The other aliquot was diluted with an equal volume of 2 $\times$ D-buffer and frozen at -20°C . After 24 h, the sample was thawed; one aliquot was dissolved and stored in electrophoresis buffer as described above (lane 2). The remaining sample of dissociated provirions was dialyzed at room temperature for 24 h against assembly buffer before electrophoretic analysis (lane 3). Aliquots containing equal numbers of counts (20,000 cpm) were subjected to electrophoresis, and proteins were visualized by autoradiography.

for Fig. 6. As seen in Table 3, the specific infectivity rose from 14% before assembly to 42% after assembly when compared with the specific infectivity of mature virions taken from the same preparation used for dissociation and reassembly. This

TABLE 3. Evidence that reassembled virions are infectious

Virus type ^a	Protein concn (mg/ml)	Infectivity (PFU/ml, 10^{10})	Specific infectivity (PFU/mg, 10^{10}) (%)
Undissociated virions (matured 24 h at room temp) ^b	0.5	7.2	14 (100)
Dissociated at pH 9	1.0	2.0	2 (14) ^c
After reassembly at pH 6.5	1.0	6.0	6 (42)

^a Provirions were purified as described in Materials and Methods and resuspended in 5 mM HEPES (pH 7). An aliquot was removed, diluted fourfold into 50 mM HEPES (pH 7)–0.1% 2-ME, and incubated for 24 h at room temperature (undissociated virions). The remainder was diluted twofold into 2 $\times$ D-buffer and incubated for 24 h at -20°C (dissociated at pH 9). After dissociation, virus was reassembled by dialysis for 24 h at room temperature against assembly buffer at pH 6.5.

^b Taken from the provirions used as starting material for the dissociation and reassembly.

^c Most, if not all, residual infectivity is due to mature virions which do not dissociate at pH 9.

FIG. 7. Sedimentation analysis of FHV particles reconstituted from purified coat protein alpha and purified FHV RNA. [^{35}S]methionine-labeled, purified coat protein alpha (200 μl ; 0.16 mg/ml) was mixed with purified, unlabeled FHV genomic RNA at a protein/RNA ratio of 5:1 (wt/wt) and dialyzed against assembly buffer for 4 h. Differentially labeled mature virions were then added, and the mixture was analyzed by sucrose gradient sedimentation as described in the legend to Fig. 5. The stippled box at fraction 30 (P) indicates that some radioactivity pelleted during centrifugation (see Fig. 5 legend).

result suggested that reassembled particles were infectious but indicated that their specific infectivity was less than that of authentic virions. We attribute this discrepancy in part to the fact that coat protein present in aggregated form after dissociation was likely unavailable for reassembly.

Reconstitution of virus-like particles from purified coat protein alpha. To sidestep complications caused by the presence of aggregated coat protein in dissociated provirions, we decided to reconstitute virus particles by using purified coat protein and purified viral RNA. Purified coat precursor alpha was obtained by separating the components of dissociated provirions on sucrose density gradients and collecting soluble alpha subunits from the top fractions (see Fig. 3 for a typical gradient profile).

Particles were reconstituted by combining alpha protein, usually at a concentration of 0.1 to 0.2 mg/ml, with viral RNA extracted from gradient-purified FHV. The components were mixed in a protein/RNA ratio of 5:1 (wt/wt), the ratio found in authentic virions. After dialysis against assembly buffer, the products were analyzed by sucrose gradient sedimentation in the presence of differentially labeled authentic FHV. The result of a typical experiment is shown in Fig. 7. In general, 50 to 70% of the protein assembled into particles that sedimented at a rate of about 130S, i.e., 5% slower than authentic virions or virions reassembled from dissociated provirions. The remaining protein formed insoluble aggregates or stayed in solution. Attempts to achieve reconstitution of particles that cosedimented with authentic virions by modifying the characteristics of assembly buffer, such as ionic strength, concentration of Ca and Mg ions, and pH, were not successful (data not shown).

Cleavage of alpha protein in reconstituted particles was normal (data not shown), but the products were essentially noninfectious. The specific infectivity of virions reconstituted

from purified coat protein and FHV RNA was 5 to 6 logs lower than that of authentic virus (data not shown).

Treatment of reconstituted particles with RNase resulted in further reduction of their sedimentation rate to about 90S (data not shown). This suggested that encapsidated RNA could be removed at least partially by exposure to RNase, possibly explaining the low infectivity usually observed for reconstituted particles. Attempts to reconstitute protein shells in the absence of RNA were not successful (data not shown).

Reconstitution of virus-like particles in the presence of heterologous RNA. Reconstitution of virus-like particles from purified coat protein alpha provided an opportunity to test whether coat protein could assemble in the presence of RNA1 or RNA2 alone and whether viral RNAs could be replaced by heterologous RNAs.

In the presence of RNA2 (Fig. 8A), assembly was efficient and particles sedimented as a sharp peak on sucrose gradients. In contrast, with RNA1 (Fig. 8B), the assembled particles sedimented as a broader peak, with sedimentation rates ranging from about 90 to 137S, suggesting the formation of more than one product.

Assembly of protein alpha also occurred in the presence of heterologous RNAs, single-stranded DNA, and dextran sulfate (Fig. 8C to F). The sedimentation rates of the assembled structures varied from about 75 to 130S. Thus, assembly of coat protein was promoted not only by FHV RNA but also by a suitable polyanion.

DISCUSSION

We have described conditions for isolation, dissociation, and reassembly of provirions from FHV. The provirions are composed of 180 capsid proteins, called alpha, and two RNAs, called RNA1 and RNA2. In infected cells, assembly of the provirion shell is fast (minutes), but the subsequent maturation cleavage, required for acquisition of infectivity, is slow (hours). Because provirions cosediment with mature virions, the two particles could not be physically separated. However, by pulse-labeling infected cells with [³⁵S]methionine for 1 h before harvesting, it was possible to isolate radiochemically pure provirions which contained a small fraction (14%) of mature virions which lacked radiolabel but which were responsible for a background level of infectivity. Because of the greater stability of mature virions, provirions could be selectively dissociated by freezing and thawing in pH 9 buffer containing 0.5 M NaCl and 0.1 M urea to yield RNA1, RNA2, and protein alpha in the monomeric state.

Divalent cations interfere with dissociability of provirions. The success of the dissociation procedure was strongly dependent on the presence of EDTA throughout the provirion purification procedure (12). Omission of the chelator at any step, including the wash preceding liberation of virus from infected cells, rendered the purified provirions refractory to dissociation. Electron density interpreted as calcium ions has been observed by X-ray crystallographic analysis of the FHV structure (6). The putative calcium ions are located at the coat subunit interfaces near the surface of the virus, where they stabilize the asymmetric unit by preventing electrostatic repulsions of neighboring carboxylate groups.

Unfractionated dissociation products spontaneously reassembled to form functional provirions with a normal complement of RNA. Upon dialysis against low-ionic-strength buffer at pH 6.5, the dissociated provirions spontaneously reassembled in high yield to form particles indistinguishable from authentic virions in sedimentation profile and sedimentation coefficient, indicating that the reassembled provirions were

homogeneous and similar in size and density to mature virions. That the population of reassembled provirions also received normal proportions of RNA1 and RNA2 was supported by electrophoretic analysis. However, the most persuasive evidence that provirions were accurately assembled was the finding that their specific infectivity increased threefold as maturation cleavage proceeded, from 14% (due to the presence of unlabeled mature virions in the preparation of purified radiolabeled provirions) to a final specific infectivity that was 42% of that of native virions. These specific infectivity values afford a powerful argument that RNA1 and RNA2 in reassembled provirions were packaged in about the same distribution as in natural provirions. For example, with a typical plaque count of 50 from a monolayer seeded with 5×10^6 cells (see reference 26), the multiplicity of infection is about 10^{-5} ; thus, the probability of a given cell being successfully coinfecting by two particles (one carrying RNA1 and the other carrying RNA2) would be in the vicinity of 10^{-10} , too low to account for the specific infectivities actually observed.

Lack of direct evidence that every nodavirus particle packages a complete genome. The evidence that reassembled particles contain both RNA1 and RNA2 does not preclude the possibility that some of the particles contain only RNA1 or only RNA2. The notion that all native virions carry one copy each of RNA1 (22S) and RNA2 (15S) is based on the observations that the buoyant density of virion populations in CsCl is homogeneous and that they contain equimolar amounts of RNAs 1 and 2 (10). The mechanism by which the correct pair of RNA molecules is packaged is not understood. One possibility is that the RNAs are packaged as a 1:1 complex, which might form via complementary bases (4) or by a linker protein (20). On the basis of the equation $S_{\text{unknown}} = S_{\text{standard}} \times (\text{MW}_{\text{unknown}}/\text{MW}_{\text{standard}})^{2/3}$ (where MW is molecular weight) and using RNA1 as the standard, such a complex was expected to sediment at about 29S. As seen in Fig. 3, however, viral RNA was resolved into two peaks representing RNA1 and RNA2. The absence of a 29S peak weakens the notion of hydrogen-bonded pairing of the genomic RNAs 1 and 2 but otherwise provides little further insight into the copackaging mechanism.

Why are provirions defective when reconstituted from separated RNA and capsid protein subunits? When particles were reconstituted from purified coat protein alpha and viral RNA, the products sedimented 5% more slowly than authentic virions and showed little, if any, infectivity. The difference in assembly behavior of fractionated and unfractionated mixtures suggests that the unfractionated dissociated products contained residual nucleating structures essential for accurate assembly. Such structures might represent discrete nucleoproteins that initiate assembly and guide accurate accretion of assembly units to form the icosahedral surface lattice. Another possibility is raised by the observation that nodaviruses, like picornaviruses, contain small, still uncharacterized, hydrophobic molecules near the fivefold vertices (16a). The functions of these "pocket factors" are still unclear and might well have an important role in assembly. Both nucleating complexes and pocket factors might have been removed during centrifugal separation of RNA and coat protein.

Preliminary attempts to identify a ribonucleoprotein in samples of dissociated provirions were inconclusive. Ultracentrifugation and gel filtration experiments indicated that the mixture of dissociated provirions contained protein aggregates, but discrete ribonucleoproteins were not detected. In addition, preliminary experiments indicate that these protein aggregates are resistant to RNase treatment (data not shown). Nevertheless, in the light of the possible importance of nucleating

FIG. 8. Sedimentation analysis of FHV particles reconstituted from purified coat protein alpha and various polyanions. [^{35}S]methionine-labeled coat protein alpha (200 μl ; 0.2 mg/ml) was mixed with unlabeled polyanions at a protein/polyanion ratio of 5:1 (wt/wt) and dialyzed against assembly buffer. After 24 h, differentially labeled mature virions were added, and the mixture was analyzed by sucrose gradient sedimentation as described in the legend to Fig. 5. The polyanions used are specified in each panel.

FIG. 9. Proposed involvement of RNA in nodavirus assembly. (i) Initiation. The initial step in nodavirus assembly is believed to involve the formation of a nucleating complex in which a coat protein substructure, e.g., a monomer or multimer of asymmetric units ("platelet"), interacts with an encapsidation signal on the viral RNA. A 14-base stem-loop in RNA2, shown to be essential for packaging (29), would be a good candidate for such an encapsidation signal. Similar stem-loops are present in RNA1 but have yet to be implicated as necessary for packaging. The existence of protein platelets, on the other hand, is suggested by the presence of discrete holes in FHV mutants, in which Asn-363 at the alpha protein cleavage was replaced by alanine (22). (ii) Propagation. Little is known about the propagation step. However, Fisher et al. (6) have shown that helical RNA duplexes containing about 10 bp interact with coat protein subunits at each of the 30 twofold axes of symmetry. Thus, it is conceivable that the initiation complex is propagated into a spherical particle by accretion of coat protein platelets which are guided into the growing shell by binding to the helical structures on the RNA.

complexes for viral assembly, the search for such structures should be repeated, perhaps by using cleavage-defective mutants. The use of such mutants, which are available (23), would eliminate possible interference by mature, nondissociable particles, whose detection on radioactivity profiles might have obscured the presence of small amounts of radioactivity representing ribonucleoprotein complexes. That nucleoprotein complexes play important roles in viral assembly has previously been demonstrated for turnip crinkle virus, a T=3 plant virus (27).

Assembly of FHV particles by either one of the two methods was dependent on the presence of RNA or, more generally, on the presence of a polyanion. This observation suggests an essential role for nucleic acid in formation of stable coat protein interactions. In fact, structural analysis of the FHV capsid by X-ray analysis has shown that the RNA is an integral component of the viral shell and controls the bonding pattern between coat subunits at the twofold-symmetry axes (6). The observation that several heterologous species of RNA and even single-stranded DNA could initiate assembly (albeit aberrant) suggests that the interactions between protein and RNA are based on structural motifs rather than on specific sequences. This seems to be a general phenomenon, since several other icosahedral RNA viruses have been reported to assemble in the presence of heterologous RNA (1, 27, 28). No attempt was made to determine if alpha protein was cleaved in these aberrantly assembled particles.

We have not yet been able to detect intermediates in the assembly of virions and may have to change the assembly conditions in order to dissect the pathway at the molecular level. The data accumulated so far suggest that assembly begins with simultaneous interactions of coat protein subunits and viral RNA, leading to rapid formation of virus particles once

an initial structure has been generated (Fig. 9). The function of RNA in the assembly process may be twofold: first, it is required as a structural component of the virion, and second, it may serve as a string that folds together as individual coat subunits or distinct oligomers are guided into the growing shell. In this case, dialysis of alpha protein in the presence of small RNA fragments should arrest assembly at an intermediate level and allow for isolation of subassembly aggregates.

Insoluble aggregates: an unresolved technical problem preventing quantitative dissociation of provirions. Dissociation of provirions into monomeric alpha chains provides convenient access to subunits required in searching for possible intermediates in the pathway of nodavirus assembly. Such intermediates may also be generated by dissociation along discrete pathways. For example, fragments generated at high ionic strength and low urea concentrations sedimented at a rate of 50 to 60S, suggesting that they might represent substructures, possibly half-shells. Further analysis of these fragments by electron microscopy may provide useful insights into nodavirus dissociation and assembly.

However, one problem which prevented quantitative reassembly of dissociated provirions was the presence of a fraction of coat protein which formed aggregates, most of which pelleted during ultracentrifugation or eluted in the void volume during gel filtration. The tendency of coat protein to aggregate might have been due to the fairly high concentration of monomeric alpha subunits, usually in the range of 0.8 to 1 mg/ml. Alternatively, it is possible that aggregation was a result of protein denaturation caused by the reducing nature of D-buffer, which contained 5 mM DTT. It has recently been shown by X-ray analysis of the FHV structure that the coat protein contains a disulfide bond formed between residues Cys-69 and Cys-318 (6). Elimination of DTT or replacement of

it with reducing agents with lower redox potential (e.g., 2-mercaptoethanol or glutathione) may eliminate this problem. It may also be useful to determine if the disulfide bonds in protein alpha are formed within the infected cell or only later after release, purification, and crystallization of virus particles.

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