

## Assembly-Dependent Maturation Cleavage in Provirions of a Small Icosahedral Insect Ribovirus

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Received 4 April 1988/Accepted 31 May 1988

Extracts from nodavirus-infected *Drosophila* cells contained detergent-labile 140S "young" particles much richer than mature virions in their content of protein alpha, a precursor of coat proteins beta and gamma. Incorporation studies in infected cells showed that most newly synthesized alpha protein was assembled into young particles within a few minutes. Incubation of the particles, either in cytoplasmic extracts or after purification, resulted in spontaneous first-order cleavage of alpha protein to form beta-plus-gamma chains. Alpha protein that was not associated with particles failed to cleave. Cleavage was accompanied by a marked increase in detergent stability of the particles and was unaffected by a broad spectrum of protease inhibitors or by coating with precipitating antibody. We conclude (i) that alpha chains are cleaved only after assembly into provirions, (ii) that cleavage occurs internally and is likely therefore autocatalytic, and (iii) that cleavage stabilizes the mature virus particles.

Viruses of the family *Nodaviridae* are attractive objects for the study of the synthesis, assembly, and structure of simple spherical virions. Black beetle virus (BBV), for example, grows exceptionally well, yielding as much as 0.1 mg/ml of infected *Drosophila* culture (12). In addition, viruses of the family *Nodaviridae* are among the smallest and simplest viruses known. The BBV genome consists of only 4,505 nucleotides (9), split between two single-stranded, messenger-active RNAs, 1 and 2. Only three gene products are known: a putative replicase protein A (104 kilodaltons [kDa]) from RNA 1; a protein B (10 kDa) of unknown function from an RNA 1-derived subgenomic RNA 3; and a coat precursor alpha protein (47 kDa) from RNA 2 (13, 14). Finally, the structure of the protein shell of BBV is now known to atomic dimensions (18), and structural studies on two other serologically distinct nodaviruses, Nodamura virus and flock house virus (FHV), are well under way.

The protein shell of mature virions is composed of 180 protomers, most of which contain two polypeptides, beta (43 kDa) and gamma (5 kDa), derived by proteolytic cleavage of a precursor protein, alpha (13). Virions also contain small but variable amounts of alpha protein. Amino-terminal sequencing of the gamma chains (18), coupled with the known nucleotide sequence of virion RNA 2 (8), showed that this cleavage of the 407-amino-acid alpha chain occurred between Asn residue 363 and Ala residue 364, indicating that gamma represented 44 relatively hydrophobic carboxy-terminal amino acids. Localization of the cleavage site does not, however, reveal the morphogenetic step at which proteolysis occurs, the protease involved, or the function of this event in the virus life cycle. We show here that alpha chains are cleaved only after assembly of the protein shell has been completed and that this cleavage is probably autocatalytic and is accompanied by an increased stability of the virus. Thus, the final step in nodavirus morphogenesis appears to be analogous to picornavirus maturation from provirions, characterized by unstable particles containing viral RNA

and an abundance of precursor 1AB chains in the capsid (11, 15, 17).

### MATERIALS AND METHODS

**Virus.** Nodavirus strain W17, which has been cloned (10), was originally thought to be a variant of BBV (26). However, cross-neutralization tests with hyperimmune antisera and cross-hybridization assays with minus-stranded W17 RNAs (T. Gallagher, Ph.D. thesis, University of Wisconsin, Madison, 1987) show that W17 virus is not BBV, but FHV, a nodavirus originally isolated from *Costelytra zealandica* larvae (25).

**Cells.** The WR subline of Schneider line 1 cells (24) was propagated in 490-cm<sup>2</sup> roller bottles (catalog no. 25130; Corning Glass Works, Corning, N.Y.) at 0.5 rpm in complete growth medium (CGM) consisting of Schneider culture medium (23) with 15% fetal bovine serum. Serum was heated for 30 min at 60°C and stored frozen at -20°C. The doubling time of the cells at 26°C was about 15 h. Cells (10<sup>8</sup> seeded into 75 ml of CGM) grew to confluence (about 2 × 10<sup>9</sup> cells per bottle) within 3 days. Suspensions were prepared, after spent medium was removed, by scraping the attached cell sheet into fresh medium with the aid of a rubber policeman.

**Infection.** Suspended *Drosophila* cells (4 × 10<sup>7</sup> cells per ml) were infected at a multiplicity of 100 PFU per cell in CGM. Cells were incubated with occasional agitation for 1 h at 26°C and then sedimented and suspended in CGM to 5 × 10<sup>6</sup> cells per ml. Infected cells were distributed into tissue culture plates, allowed to settle, and incubated without agitation at 26°C. Monolayers (2 × 10<sup>5</sup> to 2 × 10<sup>6</sup> cells per cm<sup>2</sup>) remained attached to plates until about 24 h after infection.

**Radiolabeling of intracellular RNA.** Suspensions containing 10<sup>6</sup> infected cells in 0.5 ml of CGM were seeded into wells of Falcon multititer plates (catalog no. 3047; surface area, 2 cm<sup>2</sup> per well; Becton Dickinson Labware, Oxnard, Calif.) and incubated for a few hours at 26°C to let the cells become firmly attached. Actinomycin D (0.01 ml) (0.5 mg/ml; Dactinomycin; Merck Co., Inc., West Point, Pa.) was added to each well 30 min prior to the addition of 25 to 250 µCi of [5-<sup>3</sup>H]uridine (catalog no. NET-174; New England

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Nuclear Corp., Boston, Mass.) per ml. After a 1-h incorporation period, labeling medium was carefully removed and monolayers were rinsed once with 1 ml of PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] buffer (0.035 M sodium PIPES [pH 6.8], 0.1 M NaCl, 0.01 M KCl).

Radiolabeled cell sheets were immediately suspended in TE buffer (0.01 M Tris hydrochloride, 0.001 M EDTA, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS), and then homogeneous lysates were prepared by flushing cells 5 to 10 times through a 0.2-ml Eppendorf micropipette. Samples (0.02 ml, corresponding to  $5 \times 10^4$  cells) were quickly removed and spotted onto 2.4-cm GFA glass microfiber filters (Whatman, Inc., Clifton, N.J.). After being dried at room temperature, the filters were soaked in 20% (wt/wt) trichloroacetic acid for 20 min at 0°C and then washed successively with 8% trichloroacetic acid, 95% ethanol, and diethyl ether. The disks were air dried, and radioactivity was measured by liquid scintillation spectroscopy.

**Radiolabeling of intracellular proteins.** CGM was withdrawn from each culture well, with care taken not to damage the cell monolayer, and replaced with methionine-deficient medium containing [<sup>35</sup>S]methionine (10 to 100 μCi/ml). After 1 h at 26°C, the medium was removed and cell monolayers were rinsed three times with PIPES buffer to remove serum proteins (which interfered during electrophoresis). Incorporation was halted by dissolving the cells in SDS buffer (2% SDS, 0.01 M sodium phosphate [pH 7.2] containing 1% 2-mercaptoethanol) and heating them for 5 min in a boiling water bath.

**Preparation and fractionation of provirions.** To prepare [<sup>35</sup>S]methionine-labeled cytoplasmic extracts, labeled cell monolayers ( $2.5 \times 10^5$  cells per cm<sup>2</sup>) were dispersed to  $5 \times 10^6$  cells per ml in NP40 buffer (0.1% Nonidet P-40, 0.025 M sodium phosphate [pH 7.0], 0.1 M NaCl, 0.1% 2-mercaptoethanol, 0.01% bovine serum albumin). The suspension was made homogeneous by flushing 5 to 10 times through a 0.2-ml Eppendorf micropipette. Nuclei were pelleted at room temperature ( $16,000 \times g$  for 2 min), and the supernatant was recovered.

The extracts (0.2 ml, corresponding to  $10^6$  cells) were fractionated in linear 11-ml 5 to 20% (wt/wt) sucrose gradients containing 0.025 M sodium phosphate (pH 7.0), 0.1 M NaCl, and 0.1% 2-mercaptoethanol. Centrifugation was at 40,000 rpm for 50 min at 20°C in a Beckman-Spinco SW41 rotor. Gradient fractions, typically 0.2 to 0.5 ml in volume, were collected and incubated at room temperature.

**Immune precipitation.** Virions were immunoprecipitated with antiserum and staphylococcal protein A (20). Samples (0.2 ml) of cytoplasmic extract were mixed with an equal volume of NP40 buffer containing 1% (vol/vol) rabbit antiserum raised against gradient-purified native FHV. After 10 min at room temperature, 0.1 ml of NP40 buffer containing 20% (vol/vol) hydrated protein A-Sepharose (catalog number 17-0780-01; Pharmacia, Inc., Piscataway, N.J.) was added. Incubation was continued at room temperature for 10 min, and then the Sepharose beads were pelleted at  $16,000 \times g$  for 30 s, washed five times with 1 ml of NP40 buffer each time, and suspended in 0.2 ml of NP40 buffer. The resulting washed slurry was assayed for radioactivity and incubated at room temperature.

**Electrophoretic analysis of protein.** Unless otherwise indicated, [<sup>35</sup>S]methionine-labeled proteins were electrophoresed on SDS-polyacrylamide slab gels as described previously (22). Samples (0.02 ml) in NP40 buffer were added to 0.005 ml of solubilizer solution (0.05 M sodium phosphate [pH 7.2] containing 10% SDS and 5% 2-mercaptoethanol

[21]) and heated for 5 min in a boiling water bath. Samples were diluted with an equal volume of 8 M urea–1% SDS–5% Ficoll–0.05% bromphenol blue before being loaded into wells.

Gels (14 cm by 28 cm by 0.15 cm) were prepared with 9.8% acrylamide–0.2% bisacrylamide–0.1% TEMED (*N,N,N',N'*-tetramethylethylenediamine)–0.1% SDS–5 M urea in 0.01 M sodium phosphate [pH 7.2]. Polymerization was catalyzed by the addition of ammonium persulfate to a final concentration of 0.05%. Electrophoresis was conducted at 3 V/cm with recirculation of the electrode buffer (0.01 M sodium phosphate [pH 7.2], 0.1% SDS, 0.05 M neutralized 3-mercaptopropionic acid). After electrophoresis, the gels were fixed for 4 h in an aqueous solution containing 25% methanol, 10% acetic acid, and 1% glycerol. Vacuum-dried gels were then subjected to autoradiography by using Kodak XAR film, and the distribution of radioactivity was determined by densitometry by using a Zeineh model SLR-504XL soft-laser-scanning densitometer equipped with a Zeineh Videophoresis program (Biomed Instruments, Inc., Fullerton, Calif.).

## RESULTS

**Time course of viral RNA and protein synthesis in virus-infected *Drosophila* cells.** W17 virus was chosen for study because it grows to high yield in culture (0.1 mg/ml of cell suspension), comparable to that reported for BBV (12), and because W17 virus has been molecularly cloned (10), thus facilitating future studies involving directed mutations through the methods of genetic engineering.

Viral RNA and protein synthesis were monitored in subconfluent infected cell monolayers. Under the conditions used, the cells ( $2.5 \times 10^5$ /cm<sup>2</sup>) remained firmly attached to tissue culture plates for at least 24 h, so that medium containing radiolabeled precursors could conveniently be added or removed throughout the course of the viral infection cycle. Incorporation of [<sup>35</sup>S]methionine, which was linear during each 1-h labeling period, was halted by dissolving cells in SDS solubilizer. Lysates were then subjected to electrophoresis on a polyacrylamide gel. As seen from the autoradiogram in Fig. 1b, the bulk of the radioactivity was incorporated into protein alpha which reached peak synthesis between 12 and 20 h postinfection. Quantitation of the alpha bands by densitometry is shown in the upper panel. The products of alpha cleavage, beta and gamma, were not detected during the 1-h incorporation period used in this experiment.

The faint band migrating just ahead of protein alpha (Fig. 1b) is a host protein, probably actin, whose synthesis, like that of other host proteins evident at 4 h, declines as infection progresses. Synthesis of protein B roughly parallels that of protein alpha but is less vigorous. Protein A was made in such small amounts as to be barely detectable in Fig. 1. This pattern of viral protein synthesis, with coat protein and protein B expressed vigorously and protein A produced in small amounts, is similar to that described for BBV (12, 13).

Viral RNA synthesis was not blocked by actinomycin D, an inhibitor of DNA-directed RNA synthesis. This allowed incorporation of [<sup>5-3</sup>H]uridine in actinomycin D-treated cell monolayers to reveal viral RNA synthesis rates (Fig. 1a). The rate of W17 RNA synthesis peaked at the same time as coat protein (16 h after infection) but continued for at least 28 h, in contrast to coat protein synthesis, which declined rapidly after 20 h.

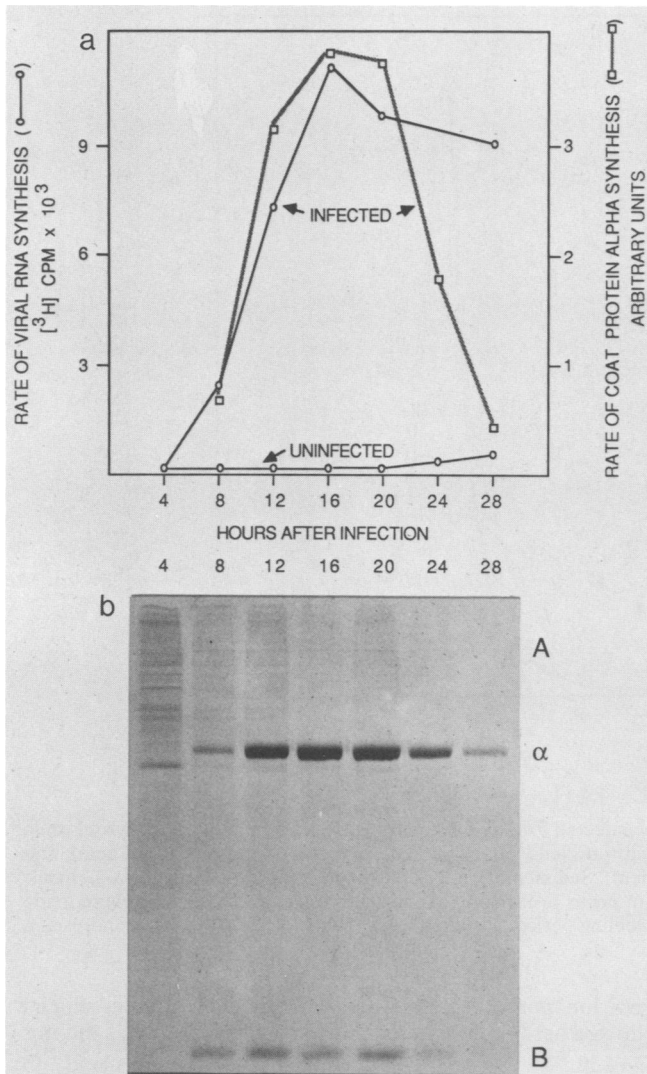


FIG. 1. (a) Time course of viral RNA and coat protein alpha synthesis in *Drosophila* cells infected with FHV (100 PFU per cell). Identical 0.5-ml samples of infected *Drosophila* cells ( $10^6$  cells per ml) were distributed into 1.6-cm-diameter tissue culture wells and incubated without agitation at 26°C. For viral RNA synthesis, 10  $\mu$ l (5  $\mu$ g) of actinomycin D was added to each well 1.5 h prior to the indicated times. After 0.5 h, 25  $\mu$ l (25  $\mu$ Ci) of [<sup>3</sup>H]uridine was added. After a 1-h labeling period, medium was removed and the adhered cell monolayer was dissolved in SDS buffer. Acid-insoluble radioactivity was determined on duplicate 20- $\mu$ l ( $5 \times 10^4$  cells) lysates by liquid scintillation spectroscopy. For coat protein alpha synthesis, 1 h before the indicated times, complete growth medium in wells was replaced with 0.5 ml of methionine-deficient medium containing [<sup>35</sup>S]methionine (100  $\mu$ Ci/ml). One hour later, the medium was removed, monolayers were rinsed, and cell sheets were dissolved in SDS buffer. (b) Electrophoretic pattern of infected-cell lysates from the above experiment. Samples representing  $10^5$  cells were electrophoresed as described previously (14). The amount of coat protein alpha synthesized during each pulse was quantified from the autoradiogram (panel b) by scanning densitometry, and the resulting values were plotted in arbitrary units (panel a).

**Cleavage of coat protein alpha in cell extracts.** The absence of cleaved protomers (containing proteins beta and gamma) in the cell lysates (Fig. 1) indicated that the time required for cleavage of precursor protein alpha was substantially longer than the 1-h incorporation period. To determine whether

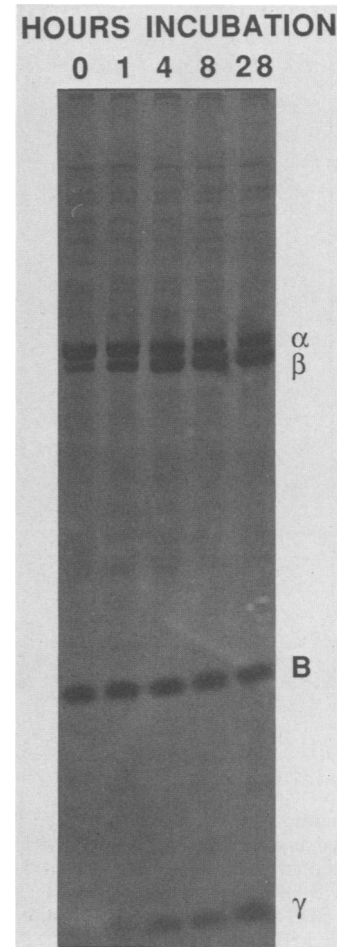


FIG. 2. Autoradiogram showing cleavage of coat protein alpha in cell extracts prepared from FHV-infected *Drosophila* culture. Cells ( $3 \times 10^6$ ) inoculated with FHV (100 PFU per cell) were radiolabeled from 15 to 16 h after infection with [<sup>35</sup>S]methionine (200  $\mu$ Ci/ml). Immediately after being labeled, cells were lysed with NP40 buffer ( $10^6$  cells per ml), nuclei were removed, and the extract was incubated at room temperature. Samples (0.02 ml) were removed at the indicated times, heated with SDS, and subjected to electrophoresis. The amount of protein A (104K) that was synthesized was too small to be detected.

cleavage of alpha would proceed *in vitro* after prolonged incubation of a cytoplasmic extract, infected cells were labeled for an hour with [<sup>35</sup>S]methionine, beginning at 15 h, the time of peak protein synthesis. After the cells were lysed with Nonidet P-40, a nonionic detergent which does not dissociate virions, the extracts were incubated at 22°C and samples were removed at intervals, heated in a boiling water bath for 5 min in SDS solubilizer, and examined electrophoretically to monitor cleavage.

The autoradiograms (Fig. 2) revealed that the alpha protein was indeed cleaved in cell extracts to form products with mobilities indistinguishable from those of the beta and gamma chains found in virions. About half of the alpha protein was cleaved within 4 h; nearly 90% of the alpha protein was cleaved by 28 h. This cleavage of alpha protein in extracts from infected *Drosophila* cells stands in sharp contrast to the behavior of alpha protein synthesized in rabbit reticulocyte extracts programmed with FHV RNA 2; such *in vitro*-translated alpha protein failed to cleave under the same conditions (data not shown).

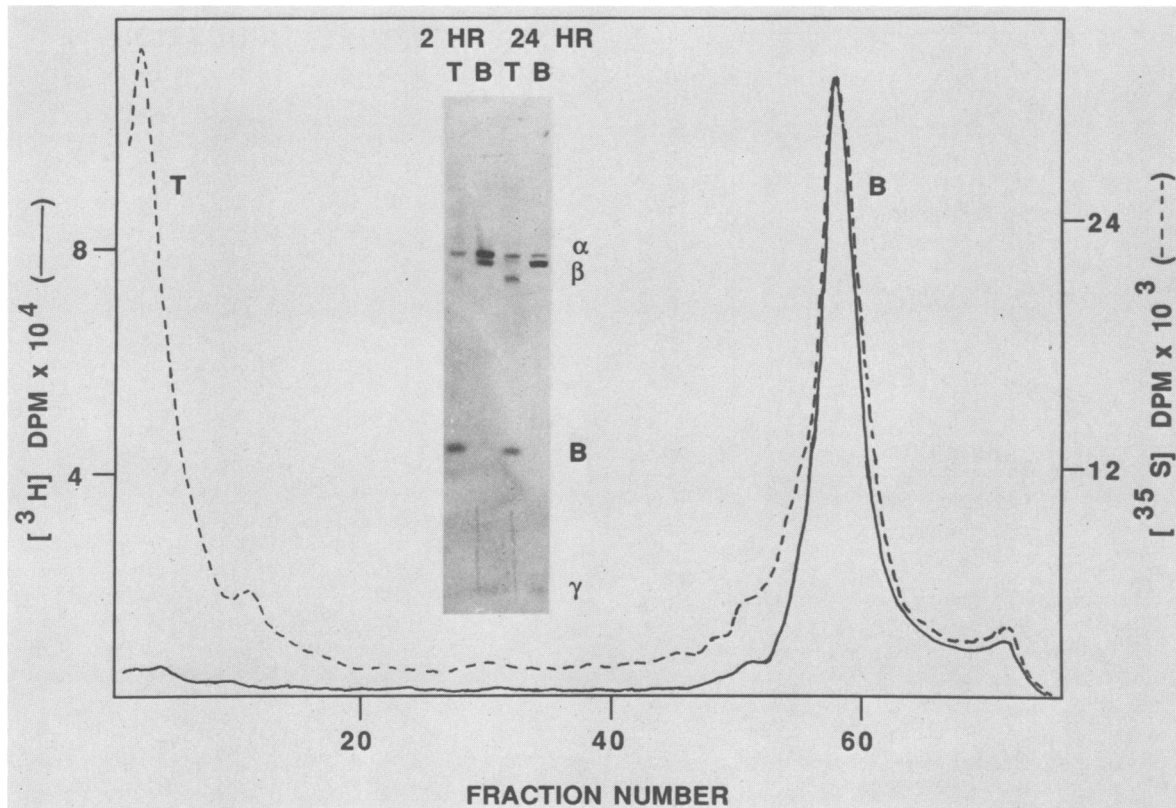


FIG. 3. Sedimentation profile of newly synthesized protein from FHV-infected *Drosophila* cells. [ $^{35}\text{S}$ ]methionine-labeled cytoplasmic extract was prepared as described in the legend to Fig. 2, mixed with gradient-purified [ $^3\text{H}$ ]uridine-labeled mature virions ( $2 \times 10^6$  cpm), and immediately sedimented through a linear 11-ml 5 to 20% sucrose gradient. Sedimentation is shown on the right vertical axis. Inset: Autoradiogram of  $^{35}\text{S}$ -labeled proteins of peaks T and B. Proteins present in peaks at the top (T) and bottom (B) of the gradient were made 2% with respect to SDS and heated at 2 or 24 h after the end of the pulse-labeling period. Proteins were then subjected to electrophoresis in SDS-polyacrylamide gels and autoradiographed.

**Dependence of alpha cleavage on assembly.** That viruslike particles were the source of the spontaneously cleaving alpha chains was shown by analyzing extracts from infected radiolabeled cells on sucrose gradients (Fig. 3). About half of the newly synthesized protein (dotted line) cosedimented with mature virions (solid line, peak B). The other half remained in peak T, near the top of the gradient.

Peak fractions from the gradient were then collected and incubated at 22°C for 2 h or 24 h, and each sample was then electrophoretically examined for cleavage of the alpha chain. As seen in Fig. 3 (inset), the bulk of the coat protein, about 90%, was found in peak B; after the 2-h incubation, less than half of the alpha protein was cleaved to beta and gamma chains, but cleavage was nearly complete by 24 h (compare inset lanes 2 and 4). The small amount of alpha protein found in peak T showed no corresponding evidence of specific cleavage to beta and gamma chains. Rather, the alpha chain found in peak T was completely intact at 2 h and appeared to have been partially degraded by 24 h to an unidentified product migrating faster than beta chains (compare inset lanes 1 and 3 in Fig. 3). The bulk of protein B, which is unrelated to coat protein, was also found in peak T. The minor peaks sedimenting between peaks T and B contained barely detectable amounts of coat protein alpha (not shown); thus, these peaks represent possible assembly intermediates which merit further attention, but they were not further examined in this study.

These results indicated that 90% of the alpha chains synthesized at 15 to 16 h were rapidly assembled into 140S

provirion particles. Cleavage to beta and gamma chains followed assembly, whereas this specific proteolysis did not occur in free coat protein.

**Rapid kinetics of provirion assembly is followed by slow maturation cleavage.** Continuing rapid synthesis of viral RNA even after decline of protein synthesis (Fig. 1) and the very rapid assembly of coat protein into virions (Fig. 3) suggested that the alpha subunit might be a limiting viral macromolecule in virion assembly. To examine this idea further, the flow of [ $^3\text{H}$ ]uridine-labeled RNA and [ $^{35}\text{S}$ ]methionine-labeled coat protein into virions was monitored from 15 to 20 h after infection. The results (Fig. 4) indicated that a 5-h chase period was required to introduce 20% of the radiolabeled viral RNA into virions, whereas 85% of the labeled coat protein was virion associated after a chase of only 10 min.

Once assembled, coat protein alpha decay was relatively slow. At 22°C (Fig. 2), the half-life of alpha chains was about 4 h, at least 25 times longer than the time required for assembly. A similar half-life was observed at 26°C and 37°C; however, at 6°C, the alpha cleavage rate was even slower—two days were required for 50% cleavage (data not shown).

The kinetics of cleavage was studied in detail at 22°C by using provirions purified by two different methods: sedimentation through a sucrose gradient or immune precipitation with neutralizing antibody. For gradient-purified virus (Fig. 5A), cleavage followed first-order kinetics for 8 h, with an alpha half-life of 3.6 h. After 8 h of incubation, when the average number of cleaved chains reached about 40 per

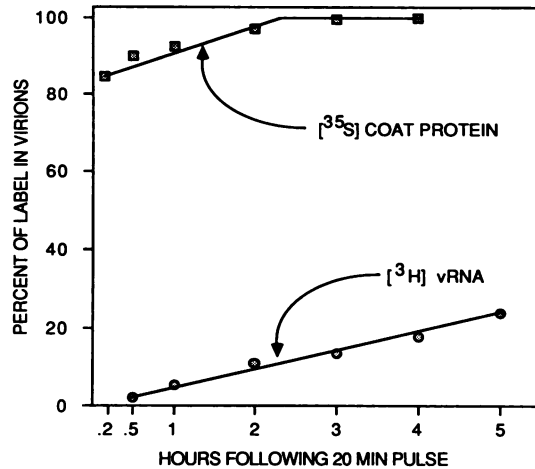


FIG. 4. Kinetics of coat protein assembly and viral RNA packaging. Identical samples of subconfluent *Drosophila* cell monolayers ( $5 \times 10^5$  cells per  $\text{cm}^2$ ), infected 15 h previously with FHV (100 PFU per cell), were labeled for 20 min with either [<sup>3</sup>H]uridine (125  $\mu\text{Ci}/\text{ml}$ ) or [<sup>35</sup>S]methionine (100  $\mu\text{Ci}/\text{ml}$ ). After labeling, monolayers were rinsed twice and then incubated at 26°C under complete growth medium containing a 100-fold excess of uridine and methionine. At the indicated times, cytoplasmic extracts were prepared from cell sheets. Immune-precipitable radioactivity was separated from the extracts, and the <sup>3</sup>H activity recovered in the precipitate was plotted as a percentage of the total <sup>3</sup>H-viral RNA synthesized during the pulse (10,000 cpm). The <sup>35</sup>S-labeled immune precipitate was allowed to incubate for 24 h at 22°C before being subjected to electrophoresis. The extent of alpha cleavage, determined from the resulting autoradiogram, was assumed to be directly proportional to the percentage of alpha association with virions. Data were corrected for the 15% uncleaved alpha protein found in mature virions.

virion, the rate of proteolysis decreased. By 24 h, the number of alpha chains reached 25 to 27 and did not thereafter decrease any further. The rate of alpha cleavage in purified particles was essentially identical to that seen in the crude extract (Fig. 2), indicating that proteases or other cytoplasmic factors removed during purification had no effect on the cleavage rate.

Isolation of these particles on sucrose gradients required about 2 h before the first sample could be measured for cleavage (Fig. 5A). Direct immune precipitation of provirions from cytoplasmic extracts could be accomplished in less than an hour. Moreover, the cleavage rate in immunoprecipitated material (Fig. 5B) was identical to that obtained by the sucrose gradient method, indicating that alpha cleavage was unaffected by antibody binding to the virus surface.

The kinetics of alpha cleavage was similarly unaffected by the addition of a wide range of protease inhibitors. Leupeptin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, *para*-chloro mercuriphenyl sulfonate, *N*-ethylmaleimide, and zinc chloride, when added to cytoplasmic extracts at 1 to 10 mM concentrations, had no effect on the cleavage of alpha chains (data not shown). These results are consistent with an inaccessible cleavage site buried deep inside the protein shell.

**Stability of provirions.** In vitro maturation of provirions was accompanied by a marked decrease in sensitivity to anionic detergents and urea (Table 1). The provirion was most sensitive to SDS, followed by sarcosyl, deoxycholate, and urea. EDTA, a chelator of divalent ions, had little apparent effect on provirion stability.

Virions matured by in vitro incubation were largely resis-

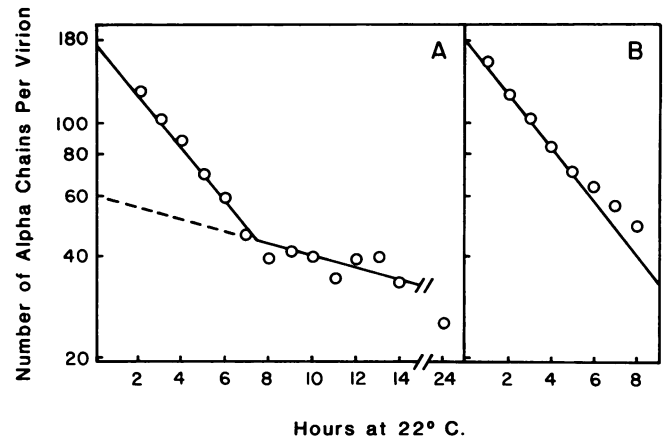


FIG. 5. Kinetics of alpha cleavage in virions at room temperature after purification by sedimentation on sucrose gradients (A) or by immunoprecipitation with neutralizing antiserum (B). Samples were removed from purified [<sup>35</sup>S]methionine-labeled virus at hourly intervals, heated in the presence of SDS, and subjected to electrophoresis. Alpha- and beta-specific radioactivity was quantified from the resulting autoradiograms by laser densitometry. The results were corrected for the alpha/beta methionine ratio (10:9) and plotted by assuming the sum of alpha and beta chains per virion to be 180.

tant to all of the agents. However, the more powerful detergents, SDS and sarcosyl, did disrupt one-third and one-fourth of the mature particles, respectively (Table 1). Analysis of the products of sucrose density gradients showed that much of the disrupted mature virus sedimented only slightly more slowly than undegraded virions, at about 100S. Electrophoretic examination of the protein and RNA in this 100S peak revealed that it contained all mature virion components except for the gamma protein, which was found at the top of the gradient (data not shown). Selective loss of the small internally located gamma protein from W17 virions thus resembles the selective loss of the small internally located VP4 protein in picornaviruses (7).

Disruption of provirions inhibited maturation cleavage, and the degree of inhibition was correlated with the extent of

TABLE 1. Effect of denaturants and EDTA on the stability of provirions

Treatment <sup>a</sup>	% Radioactivity remaining in 140S particles	
	Provirion	Mature virion
None (dH <sub>2</sub> O)	100	100
1% SDS	4	66
1% sarcosyl	11	77
1% deoxycholate	15	97
1 M urea	21	98
20 mM EDTA	80	99
10 mM MgCl <sub>2</sub> + 1% SDS <sup>b</sup>	72	100
10 mM CaCl <sub>2</sub> + 1% SDS <sup>b</sup>	98	100

<sup>a</sup> 0.02 ml of the reagents at 10 times the indicated concentration was added to 0.18 ml of freshly prepared cytoplasmic extract (pH 6.8) representing  $10^6$  [<sup>35</sup>S]methionine-labeled cells prepared as described in the legend to Fig. 2. Mature virions were prepared by incubating the extracts for 24 h at room temperature before treatment. Treated extracts were analyzed for surviving 140S particles by sedimentation of each sample through a linear 11-ml 5 to 20% sucrose density gradient. Radioactivity in each peak was normalized to that found in the control sample (56,000 dpm). dH<sub>2</sub>O, Distilled water.

<sup>b</sup> Divalent cations were added at the indicated concentrations to freshly prepared extracts, and sensitivity of 140S particles to 1% SDS treatment was tested immediately thereafter (provirion) and after a 24-h incubation (mature virion) as described in the text.

TABLE 2. Effect of denaturants and EDTA on the cleavage of alpha chains in cell extracts

Treatment <sup>a</sup>	% of alpha chains cleaved
None (dH <sub>2</sub> O).....	54
1% SDS.....	0
1% sarcosyl.....	11
1% deoxycholate.....	21
1 M urea.....	24
20 mM EDTA.....	36

<sup>a</sup> Cytoplasmic extracts, prepared as described in Table 1, footnote a, were incubated for 8 h at 22°C. Portions of each sample were then subjected to electrophoresis. The extent of alpha chain cleavage was determined by densitometric analysis of the resulting autoradiogram. dH<sub>2</sub>O, Distilled water.

disruption (Table 2). This observation supports the idea that cleavage occurs only after virions have been assembled.

**Effect of calcium and magnesium on detergent lability of provirions.** Provirion sensitivity to detergent was observed only in the absence of calcium and magnesium. Addition of CaCl<sub>2</sub> (0.01 M) rendered both provirions and mature virions completely resistant to 1% SDS treatment (Table 1). MgCl<sub>2</sub> (0.01 M) had a similar but slightly less potent stabilizing effect. Thus, these divalent cations, which are known to play key roles in the stabilization of both plant (4, 19) and animal (5) viruses, also serve a similar function in stabilizing this insect virus.

## DISCUSSION

We have shown here that cleavage of nodavirus coat precursor alpha occurs only after the production of the virion particle; unassembled alpha protomers did not cleave, although they may have suffered some nonspecific degradation when incubated in cell extracts. Maturation cleavage was accompanied by an increase in stability of the virion. The final step in morphogenesis of nodaviruses is similar in both respects to maturation of provirions in picornaviruses (11, 15). The ability of detergent to selectively dislodge gamma protein from nodavirus particles matured in vitro from provirions is also reminiscent of the selective loss of VP4 during denaturation or uncoating of mature picornavirions (7).

**Nodavirus morphogenesis.** A model illustrating our current understanding of nodavirus synthesis and assembly is shown in Fig. 6. Provirion assembly occurred very quickly; 85% of newly synthesized coat protein is directed into provirions within 10 min. The rate of virion RNA encapsidation, on the other hand, is relatively slow. Thus unpackaged RNA accumulates in infected *Drosophila* cells, but coat precursors do not. It is not surprising, therefore, that viral RNA synthesis continues at a high rate for hours after peak production of coat protein (Fig. 1). The lack of any prominent [<sup>35</sup>S]methionine-labeled peaks in the sucrose gradient (Fig. 3) is consistent with rapid provirion assembly and probably reflects a short life of intermediates in vivo. This pattern of viral macromolecule synthesis differs from that seen in picornavirus-infected cells (2, 3), where newly synthesized RNA appears to be the limiting factor and coat precursors accumulate appreciable pools of intermediates such as 14S pentamers and 70S empty capsids.

**Do biphasic cleavage kinetics betray differences in folding architecture in one of the three identical chains which constitute the asymmetric unit?** The slow rate of maturation cleavage, with a half-life of 3.6 h at 22°C, contrasts sharply with the rapidity of provirion assembly. This has provided a

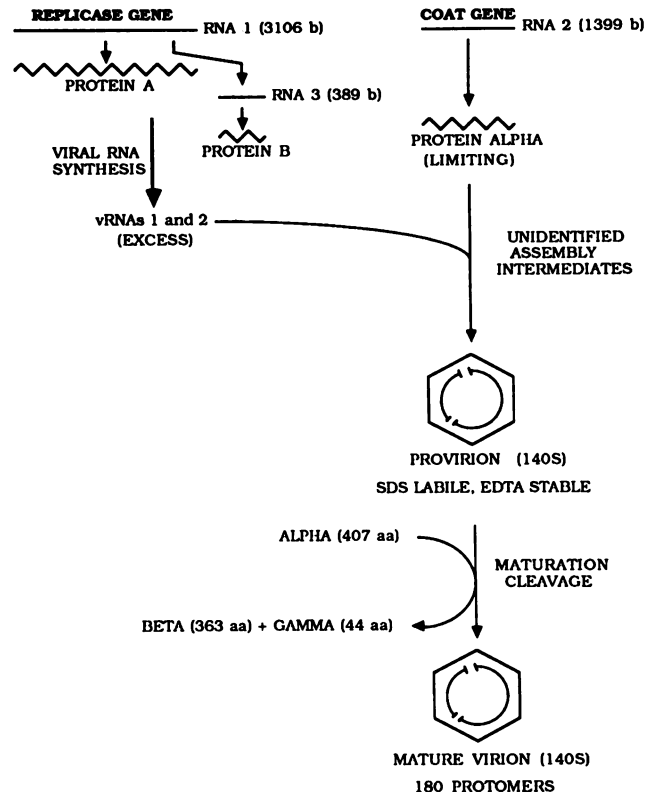


FIG. 6. Model illustrating our current understanding of nodavirus morphogenesis. Coat protein precursor alpha is synthesized in limiting amounts relative to virion RNAs 1 and 2. Maturation cleavage begins after provirion assembly and generates stable mature virions. Protomer refers to protein alpha or its cleavage product (beta plus gamma).

unique opportunity to isolate provirions in quantity sufficient for analysis of cleavage kinetics. Two stages were observed. In an early stage, about 8 h long, about two-thirds of the chains were cleaved with first-order kinetics; this was followed by a second stage with much slower cleavage of about half of the remaining chains. One explanation is that the virus population contained a minor fraction of maturation-defective particles.

A much more interesting possibility is that this biphasic curve reflects structural differences in folding of the tails in protomers occupying the three quasiequivalent domains of the 60 asymmetric units that make up the 180-subunit surface (Fig. 7). The principles of icosahedral symmetry require that identical proteins within the asymmetric unit cannot occupy identical positions (6). With BBV, this condition is accommodated at least in part by differences in folding of protomer tails (Fig. 7). Thus, the carboxy-terminal tail of a protomer at the quasiequivalent C-domain is tucked into a hinge at the B-joints (60 per virion), while the tails of the protomers at the S-joints (120 per virion) are not. Rapid cleavage of two-thirds of the chains would be consistent with the idea that tails at the two S-joints are cleaved more rapidly than the tail at the B-joint. Further insight into this issue, as well as the role of cleavages in shell stabilization, will undoubtedly emerge from crystallographic analysis of provirion particles containing only uncleaved coat protein.

**Evidence that maturation cleavage is autocatalytic.** A number of facts argue that the nodavirus maturation cleavage is accomplished autocatalytically by coat protomers. First,

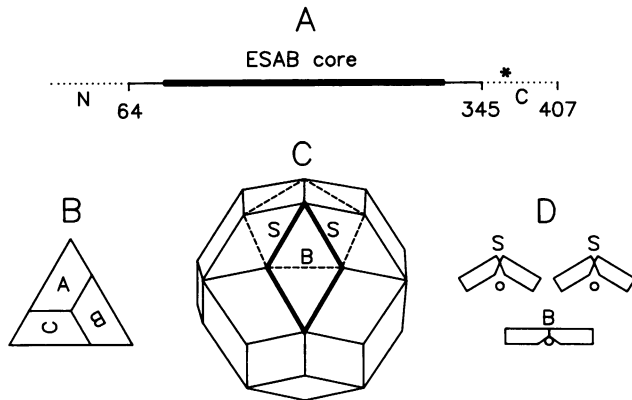


FIG. 7. (A) Location of the cleavage site between Asn363 and Ala364 (\*) in the 407-amino-acid coat protomer of BBV. The eight-stranded antiparallel beta barrel (heavy line), which makes up the main body (ESAB core) of the protein shell, extends from residues 92 to 310. Dashed segments, N (arm to the left of residue 64) and C (arm to the right of residue 345), lie inside the shell near the RNA, but their precise locations and folding patterns have not yet been resolved in the crystallographic electron density map (18), indicating that these arms are not as uniformly ordered as other regions of the protomer. (B) The asymmetric unit. Each of the 60 triangular asymmetric units constituting the protein shell of the virus contains three protomers presumably identical in sequence but not quite identical in packing because each protomer lies in a distinct quasiequivalent domain (A, B, and C). (C) Rhombic triacontahedron illustrating how each asymmetric unit abuts three neighbors. Asymmetric units joined through the two sides (S-joint) are bent  $44^\circ$  to form a fivefold vertex (dotted outline), while asymmetric units joined at the base (B-joint) lie in a plane (18) to form a flat diamond-shaped surface (bold outline). (D) Side view of hinges at the S- and B-joints between asymmetric units. Note that there are two S-joints for each B-joint. The B-joint is prevented from bending by an internal segment of protein (probably residues 376 to 407) which folds into the hinge of the B-joint but not into the hinges of the S joints (18). The presence of an arm at the B-joint, but not at the S-joints, is also observed in tomato bushy stunt virus (16) and southern bean mosaic virus (1).

crystallographic evidence suggests that the cleavage site is buried deep within the virion shell near the RNA core. Second, virions appear to contain no proteins other than coat protein (see, e.g., Fig. 1 in reference 18). Third, purified provirions cleaved as rapidly as those in cytoplasmic extracts, even in the presence of protease inhibitors. Were cleavage carried out at the virion surface by an external protease, then purification of the virions would have been expected to slow or block the process. Finally, internalizing an initially external cleavage site would seem to require a major reorientation of the protomer; not only is it difficult to conceive of a mechanism for such a major reorientation, but the finding that neutralizing antibody had no discernible effect on the cleavage rate of purified provirions (compare panels A and B in Fig. 5) further argues against the internalization hypothesis.

**Is maturation cleavage required for virion infectivity?** It is generally assumed that maturation cleavage is essential for acquisition of infectivity, but a critical test of provirion infectivity remains to be performed for both picornaviruses and nodaviruses. For FHV, provirion infectivity could not be decisively tested because there is no known way to arrest the conversion of provirions to their mature form. However, a solution to this problem may be available through recombinant DNA methodology. Infectious transcripts derived

from cDNA clones of the W17 genome have been produced (10), and site-specific mutagenesis of these clones at regions encoding amino acid residues flanking the beta-gamma cleavage site is currently under way. Such mutant RNAs, when introduced into *Drosophila* cells sensitized to free RNA infection, may induce formation of provirions incapable of maturing. If so, the issue of provirion infectivity may soon be resolved.

#### ACKNOWLEDGMENT

This work was supported by Public Health Service grant R37 AI22813 from the National Institutes of Health.

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