JOURNAL OF VIROLOGY, Nov. 1992, p. 6728–6734
0022-538X/92/116728-07$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 11

Maturation Cleavage Required for Infectivity of a Nodavirus

ANETTE SCHNEEMANN, WEIDONG ZHONG, THOMAS M. GALLAGHER,† AND ROLAND R. RUECKERT*

Institute for Molecular Virology and Department of Biochemistry, The Graduate School and College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706-1596

Received 15 July 1992/Accepted 13 August 1992

Nodaviral morphogenesis involves formation of labile precursor particles, called provirions, which mature by autocatalytic cleavage of the 407-residue coat precursor protein between asparagine residue 363 and alanine residue 364. It has previously been demonstrated that maturation results in increased physicochemical stability of the virion. We show here that cleavage of coat protein in purified provirions of Flock House virus was accompanied by a five- to eightfold increase in specific infectivity. Cleavage-negative provirions, produced by site-directed mutagenesis of asparagine residue 363 to aspartate, threonine, or alanine, displayed no infectivity above revertant frequencies as measured by plaque assay. All viable revertants (nine of nine) restored asparagine to the mutated position, suggesting high specificity for asparagine at the cleavage site.

Nodaviruses are a family of nonenveloped icosahedral insect viruses with a bipartite RNA genome (for a review, see reference 15). Members include at least four serotypes (10), represented by Nodamura virus (25), black beetle virus (18), Flock House virus (FHV) (6), and Boolarra virus (21). They are among the simplest viruses known and serve as attractive models for studies of viral replication, structure, and evolution. The simplicity is reflected in the small genome, which consists of two single-stranded mRNA molecules (4, 5, 7), both encapsidated in the same particle (19, 20, 26). RNA1 (3.1 kb), which carries all the viral information required for RNA transcription and replication (11), encodes two proteins, protein A (102 kDa) (14, 24) and protein B (10 kDa) (8). Protein B is expressed from a subgenomic RNA3 (0.4 kb) derived from the 3′ end of RNA1 (8, 13). RNA2 (1.4 kb) encodes coat precursor protein alpha (43 kDa) and has an additional function in regulating the synthesis of RNA3 (2, 3, 7, 9). RNA1 replicates independently of RNA2 in infected cells; for synthesis of virus particles, however, both RNAs are required (11).

The major protein produced during viral infection is coat precursor protein alpha (7). Synthesis of protein alpha peaks at about 15 h and is followed by rapid assembly of provirions (12). Provirions are labile precursor particles that mature by autocatalytic cleavage of the 407-amino-acid alpha chain between asparagine 363 and alanine 364 (16). The cleavage products, proteins beta (38 kDa) and gamma (5 kDa), remain part of the mature virion (16). Compared with provirion assembly, which is complete within minutes, cleavage of protein alpha is very slow. The half-life of provirions at growth temperature (26°C) is 4 h and can be prolonged to 2 days by chilling (12). The slow turnover results in temporary accumulation of provirions in infected cells after synthesis of coat protein alpha reaches maximum levels.

X-ray crystallographic determination of the three-dimensional structure of mature black beetle virus particles has revealed that the protein shell is composed of 180 protomers (16). Most protomers contain one copy each of proteins beta and gamma, although a few remain present in the alpha state. The cleavage site is located inside the virion near the RNA core (16) and is inaccessible to proteinase inhibitors and virus-precipitating antibodies (12). A method to arrest maturation cleavage is therefore not available. The first insights regarding the possible functions of provirion maturation came from physicochemical analyses which demonstrated that cleavage is accompanied by an increase in virion stability (12).

Availability of molecular cDNA clones of FHV genomic RNAs (5) provided an opportunity to examine the role of maturation cleavage in the acquisition of virion infectivity. Here, we show that replacement of asparagine 363 by aspartate, threonine, or alanine resulted in synthesis of cleavage-defective provirions which displayed no measurable infectivity by plaque assay. Viable revertants emerged at low frequencies and restored asparagine to the previously mutated position. These results suggest that maturation is required for virion infectivity and that asparagine 363 plays a crucial role in the cleavage mechanism.

MATERIALS AND METHODS

Propagation of FHV. FHV was propagated in *Drosophila* cells as described elsewhere (12). For preparation of radiolabeled virus, medium was removed from cells at 15 h postinfection and 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]). Cells were covered with 5 ml of methionine-deficient Grace's insect medium (GIBCO Laboratories) complemented with 50 μCi of [^{35}S]methionine (Amersham [catalog no. SJ.1015]) and incubated for another hour at 26°C before isolation of provirions.

Purification of provirions. At 16 h postinfection, medium was removed from cells and monolayers were rinsed three times with 10 ml of ice-cold HE buffer (0.1 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7], 10 mM EDTA, 0.1% 2-mercaptoethanol [2-ME], 0.01% BSA). Tissue culture plates were then placed on ice before lysis of cells in ice-cold HE buffer containing 1% (vol/vol) Nonidet P-40 (NP-40). The lysates were centrifuged at top speed in a tabletop centrifuge for 5 min at 4°C to remove nuclei and cell debris. Virus in 1-ml extracts was pelleted

* Corresponding author.
† Present address: Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University of Chicago, 2160 South First Avenue, Maywood, IL 60153.

through a linear 2-ml volume of 10 to 30% (wt/wt) sucrose gradients in HE buffer lacking BSA. A Beckman ultracentrifuge equipped with a TLA-100.3 fixed-angle rotor was used for this purpose. Centrifugation was at 100,000 rpm for 13 min at 5°C. Pellets were resuspended in 50 mM HEPES (pH 7)–0.1% 2-ME containing 10 mM $CaCl_2$ or 5 mM $CaCl_2$–5 mM $MgCl_2$. Divalent cations were included in the buffer for stabilization of provirions (12). Yields from a 100-mm tissue culture plate containing 10^8 cells were typically 0.8 to 1.0 mg.

Electrophoretic analysis of proteins. Electrophoresis was performed on discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels according to the procedure described by Laemmli (17). Protein samples were combined with 5× sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-ME, 0.1% bromophenol blue) and heated in a boiling water bath for 10 min. Slab gels contained 12% (wt/vol) acrylamide in the resolving gel and 5% (wt/vol) acrylamide in the stacking gel. After electrophoresis at 150 V for 6 h, the gels were fixed and stained with Coomassie brilliant blue according to standard procedures.

Plaque assay. Infectivity titers were determined on monolayers of *Drosophila* cells as described previously (26), with two modifications. First, SeaPlaque agarose was replaced with 1% low-melting-point agarose (Bethesda Research Laboratories), which can be dissolved directly in complete growth medium by being incubated for 10 min at 65°C; some lots contained insoluble particulate material which did not interfere with the assay. Second, a tetrazolium dye, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, Mo.), was used instead of crystal violet to stain monolayers. MTT solution was prepared by dissolving MTT to 3 mg/ml in water, and 0.5 ml was pipetted directly onto the overlay at 50 h postinfection. This procedure enables plaques to be visualized without killing virus in the overlay.

Site-directed mutagenesis of coat protein cleavage site. For oligonucleotide-directed, in vitro mutagenesis, a DNA fragment containing the full-length cDNA clone of FHV RNA2 was released from plasmid p2B10SP (5) by digestion with *Bam*HI and *Hin*dIII and subcloned into the *Bam*HI and *Hin*dIII sites of pBluescript II KS(+) (Stratagene, La Jolla, Calif.). Following transformation of *Escherichia coli* JM101 and superinfection with helper phage M13K07, single-stranded DNA was isolated from polyethylene glycol-precipitated phage by extraction with phenol-chloroform and ethanol precipitation.

Mutagenizing oligodeoxynucleotides consisting of 21 bases were synthesized at the University of Wisconsin—Madison Biotechnology Center. Their sequences and orientations relative to the coding strand, together with the specific amino acid change that each one introduces at position 363 in protein alpha, are as follows (mispaired bases are in lowercase letters): Asn-363→Asp, 3′-CGC CGG GTT cTA CGT AGT TAC-5′; Asn-363→Thr, 3′-CGC CGG GTT TgA CGT AGT TAC-5′; and Asn-363→Ala, 3′-CGC CGG GTT cgA CGT AGT TAC-5′. In vitro mutagenesis was performed with a mutagenesis kit from Amersham (catalog no. RPN.1523) according to protocols provided by the manufacturer. Mutant sequences were confirmed by the dideoxy-sequencing method (23) by standard procedures. Fragments of the cDNA clones containing the desired mutations were excised from pBluescript II KS(+) by digestion with *Cla*I-*Xba*I and used to replace the homologous region in plasmid p2BS(+). p2BS(+) contains a full-length wild-type cDNA copy of RNA2 downstream of the T3 RNA polymerase promoter and was used for in vitro transcription of RNA2.

In vitro transcription of RNA2. Plasmid p2BS(+) and its mutant versions were linearized with *Xba*I and used as templates for transcription with T3 RNA polymerase. Specifically, transcription reactions were carried out in a total volume of 100 μl containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 100 U of RNasin (Promega, Madison, Wis.), 0.5 mM (each) ATP, CTP, and UTP, 0.05 mM GTP, 0.5 mM sodium diguanosine triphosphate [G(5′)ppp(5′)G] (Pharmacia LKB Biotechnology, Piscataway, N.J.), 2 μg of linearized template DNA, and 100 U of T3 RNA polymerase (Promega). After incubation of the reaction mix at 37°C for 2 h, template DNA was digested by the addition of 2 U of RQ1 RNase-free DNase (Promega) and incubation at 37°C for 15 min. Transcripts were purified over Qiagen tip 20 columns (Qiagen Inc., Studio City, Calif.) according to the manufacturer's instructions. Each full-length transcript contained four nonviral bases at the 3′ end (5′-CTAG-3′). No extra bases were present at the 5′ end.

Purification of FHV virion RNA1. Total virion RNA (vRNA), extracted from gradient-purified FHV, was incubated at 65°C for 5 min and quick-chilled in ice water. RNA1 and RNA2 were then separated on a horizontal 1% low-melting-point agarose gel (10 by 10 by 0.5 cm) in Tris-borate-EDTA at 75 V for 3 h and then stained by ethidium bromide. The band corresponding to RNA1 was excised and heated to 65°C to melt the agarose. The molten agarose was loaded into slots of a second 1% low-melting-point agarose gel, and RNA1 was electrophoresed as described above. This procedure was repeated once more. RNA1 was then purified from the gel by extraction with phenol-chloroform following standard procedures. Yields of about 10 μg of RNA1 were obtained from 100 μg of total virion RNA. The stock was estimated to be enriched by at least 10,000-fold in RNA1.

To eliminate remaining traces of RNA2 in this preparation, RNA1 was selectively amplified in *Drosophila* cells by adaptation of a procedure previously described for purification of Nodamura virus RNA1 (1). A 20-ng sample of gel-purified vRNA1 was mixed with 20 ng of in vitro-synthesized negative-strand RNA2 (24) in a total volume of 30 μl and heated to 65°C for 5 min. After cooling to room temperature for 15 min, the RNA was combined with 20 μl of lipofectin (1 mg/ml; Bethesda Research Laboratories) and 10 μl of RNase-free water. The entire mixture was inoculated into 10^7 *Drosophila* cells (about 1,000 RNA1 molecules per cell) as described below under "Liposome-mediated transfection of *Drosophila* cells." At 18 to 20 h posttransfection, medium was removed from the cells and the monolayers were washed once with 2 ml of phosphate-buffered saline lacking calcium and magnesium. The cells were lysed in Tris–sodium chloride–EDTA–0.1% SDS, and total RNA was extracted twice with phenol-chloroform. Purified RNA was denatured for 5 min at 65°C, quick-chilled in ice water, and electrophoresed on a 1% agarose gel in Tris-acetate-EDTA buffer for 1.5 h at 75 V. After being stained with ethidium bromide, the band corresponding to RNA1 was excised and RNA was purified by using the RNaid kit (Bio 101, Inc., La Jolla, Calif.). The purified RNA1 sample, called passI, was analyzed for RNA2 contamination by testing lysates of cells inoculated with the sample for the presence of infectious virus. Specifically, 10^7 *Drosophila* cells were transfected with 100 ng of RNA1, incubated for 18 h, and lysed with NP-40 at a final concentration of 0.5%. Nuclei were removed by centrifugation of the cell lysate at the highest speed in a

Sorvall tabletop centrifuge (model Microspin 24S) for 10 min, and virus in the 2-ml extract was concentrated by pelleting through a 1-ml volume of a 30% (wt/wt) sucrose cushion in 50 mM HEPES (pH 7)–0.1% 2-ME–0.1% BSA with a Beckman ultracentrifuge equipped with a TLA-100.3 rotor. Centrifugation was at 100,000 rpm for 13 min at 5°C. The pellet was resuspended in 50 mM HEPES (pH 7)–0.1% 2-ME, and the infectivity titer of the suspension was determined by plaque assay. The cell amplification procedure was repeated with passI and passII, etc., until virus was not detected anymore.

Liposome-mediated RNA transfection of *Drosophila* cells. Transfections were carried out in six-well tissue culture plates (Falcon [no. 3046]) containing 10^7 cells per well. To remove serum components from cells, monolayers were washed three times with 2 ml and were then covered with 1 ml of serum-free medium. RNA-liposome complexes for 10^7 cells were prepared in the following manner. A 20-μl volume of lipofectin (Bethesda Research Laboratories) at a concentration of 1 mg/ml was diluted to 30 μl with 10 μl of water in a polystyrene tube (Falcon [no. 2054]). A mixture of 400 ng of RNA containing 200 ng of vRNA1 and 200 ng of transcript RNA2 in 30 μl of water was added. The complexes were allowed to form by incubation at room temperature for 15 min and were then applied directly to the cells. After a 1.5-h incubation period at 26°C, the medium was removed from the cells and replaced with 2 ml of complete growth medium. Incubation was continued for 16.5 h.

Purification of virus particles from transfected cells. At 18 h posttransfection, the medium was removed from each well and the cells were lysed in 100 mM HEPES (pH 7) containing 0.5% (vol/vol) NP-40, 0.01% (wt/vol) BSA, and 0.1% (vol/vol) 2-ME. After incubation on ice for 10 min, cell debris was pelleted in an SS34 rotor at 10,000 rpm for 10 min at 4°C. RNase A was added to the supernatant to a final concentration of 18 μg/ml, which was then incubated at 26°C for 30 min. Material precipitating during this time was removed by centrifugation at 10,000 rpm in an SS34 rotor for 10 min at 4°C. Virus particles in the supernatant were pelleted through a 1-ml volume of 30% (wt/wt) sucrose cushion in 50 mM HEPES (pH 7)–0.1% 2-ME–0.1% BSA at 40,000 rpm in an SW41 rotor for 2.5 h at 6°C. The pellet was resuspended in 0.5 ml of 50 mM HEPES (pH 7)–0.1% 2-ME. Virus was sedimented through a 5 to 20% (wt/wt) sucrose gradient in 50 mM HEPES (pH 7)–0.1% 2-ME at 40,000 rpm in an SW41 rotor for 1 h at 11°C. The virus band, detectable by light scattering, was harvested by removing 0.4-ml fractions from the top of the gradient.

RESULTS

Purification of provirions. Provirions were harvested at 16 h, about an hour after maximum synthesis of viral coat protein (12). To minimize spontaneous cleavage of protein alpha, the purification procedure was designed for speed and all operations were carried out in the cold; the entire procedure from harvest to purified particles was usually complete within 45 min.

Provirions prepared in this way displayed a single homogeneous peak of radioactivity sedimenting at a rate indistinguishable from that of mature 137S FHV virions (Fig. 1). Electrophoretic analysis on Coomassie-stained SDS-polyacrylamide gels showed that >80% of the coat protein was present as alpha chains (Fig. 2, lane 2). The remaining 20% represented background contamination by cellular proteins but also included small amounts of beta chains. This indicated that the provirion preparation contained low levels of mature or partially matured virus particles. As reported previously (12), alpha protein in provirions cleaved spontaneously at room temperature to beta and gamma, with cleavage being 90% complete at 24 h (Fig. 2, lane 3). Cleavage product gamma (5 kDa), which stains poorly with Coomassie brilliant blue (7), was not visible on the gel.

Maturation cleavage is accompanied by increase in virion infectivity. Cleavage of protein alpha in provirions was accompanied by a substantial increase in specific infectivity

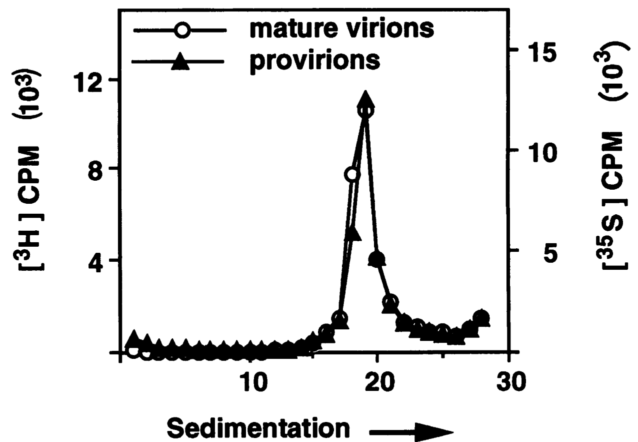

FIG. 1. Sedimentation profile of provirions isolated from FHV-infected *Drosophila* cells. [^{35}S]methionine-labeled provirions (10^5 cpm) were resuspended in 50 mM HEPES (pH 7)–0.1% 2-ME–10 mM $CaCl_2$ and mixed with gradient-purified [^{3}H]uridine-labeled mature virions (5×10^4 cpm). The mixture (100 μl) was immediately 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 and counted after 200 μl of water and 5 ml of scintillation fluid (Ecoscint) were added to each fraction. Recovery of radioactivity applied to the gradient was about 60%.

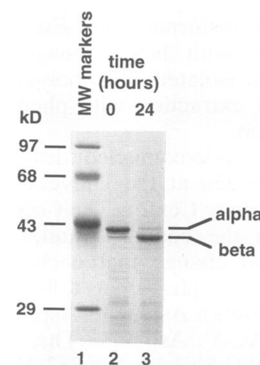

FIG. 2. Electropherogram showing cleavage of coat protein alpha in purified provirions following incubation at room temperature. Provirions, purified as described in Materials and Methods, were divided into two aliquots. One aliquot was immediately diluted with 5× electrophoresis buffer, and the dilution was heated in a boiling water bath and stored frozen pending analysis (lane 2). The other aliquot was incubated for 24 h at room temperature before electrophoresis (lane 3). Proteins were visualized by staining with Coomassie brilliant blue. Cleavage product gamma (5 kDa), which stains poorly with Coomassie brilliant blue, is not visible on this gel. MW, molecular mass.

TABLE 1. Effect of maturation on acquisition of infectivity[a]

Expt no.	Incubation of provirions at room temperature (h)	Specific infectivity of provirions (10^{10} PFU/mg)	% Infectivity[b]
1	0	2.5	10
	24	20.0	81
2	0	4.0	14
	24	21.0	72

[a] Provirions were resuspended in 50 mM HEPES (pH 7)–5 mM $CaCl_2$–5 mM $MgCl_2$–0.1% 2-ME–0.1% BSA (maturation buffer). After spectrophotometric determination of provirion concentration, the sample was diluted with maturation buffer to a particle concentration identical with that of a mature FHV stock. Provirions, kept chilled on ice during these operations, were then divided into two aliquots. One aliquot was plated for infectivity with minimum delay (i.e., within 1 to 2 h after resuspension of the provirion pellet). The other aliquot was incubated at room temperature for 24 h and then assayed for infectivity.

[b] Relative to specific infectivity of a reference preparation of gradient-purified mature virions, tested in parallel (25×10^{10} PFU/mg in experiment 1; 29×10^{10} PFU/mg in experiment 2).

TABLE 2. Steps in preparation of vRNA1 free of vRNA2

vRNA1 preparation	Yield after amplification (μg)[a]	PFU recovered from inoculated *Drosophila* cells[b,c]
Electrophoretically purified vRNA1 (three cycles)	NA[d]	1.0×10^7
Amplified passI	0.7	8.8×10^4
Amplified passII	1.0	<40[e]
Amplified passIII	7.8	<40[e]
Amplified passIV	90.0	<40[e]

[a] From 10^7 cells in passI and passII; from 3×10^7 cells in passIII; from 7×10^7 cells in passIV. See Materials and Methods for details.

[b] *Drosophila* cells (10^7) were inoculated with 100 ng of the respective vRNA1 sample and incubated for 18 h. Cell lysates were tested for presence of infectious virus by plaque assay as described in Materials and Methods.

[c] Each PFU corresponds to 300 particles (1 μg of FHV = 6.37×10^{10} particles).

[d] NA, not applicable.

[e] No plaques detected.

(Table 1). When expressed relative to a control of mature virus particles, tested in parallel, specific infectivity of provirion preparation 1 rose from a background level of 10% at zero time to 81% at 24 h. With a second preparation, infectivity rose from 14 to 72%. These results were the first indication that maturation cleavage played a role in the acquisition of particle infectivity and suggested that provirions were appreciably less infectious than mature particles. Contamination of the provirion preparation by mature virions (Fig. 2, lane 2) precluded definitive conclusions about the specific infectivity of pure provirions; nonetheless, these results indicated that they were at least five- to eightfold less infectious than mature virions.

To determine whether the increase in infectivity was due to more-efficient binding of mature virions to the cellular receptor, we tested attachment of [^{35}S]methionine-labeled provirions to *Drosophila* cells before and after incubation at room temperature. A 1.8-fold increase in attachability was detected for mature virions, but the effect was too small to explain the difference in specific infectivity (data not shown).

Synthesis of virus particles by RNA transfection of *Drosophila* cells. To further analyze the role of maturation in the acquisition of virion infectivity, we set out to produce genetically engineered mutant viruses containing alterations in the beta-gamma cleavage site. This was initially attempted by RNA transfection of *Drosophila* cells with in vitro-synthesized transcripts of RNA1 and RNA2 (trRNA1 and trRNA2). The use of trRNA2 permitted introduction of amino acid changes at the cleavage site of coat protein alpha via site-directed mutagenesis of its cDNA clone. However, preliminary experiments with wild-type transcripts showed that the trRNAs, unlike authentic vRNAs, failed to promote the synthesis of satisfactory levels of virus (see below). Through the use of reassortments of trRNAs and vRNA1 and vRNA2, we established that this low yield was attributable to trRNA1, an RNA that contained four nonviral bases at the 3′ end. We therefore decided to use vRNA1 and mutant trRNA2 to synthesize altered virions. vRNA1 preparations devoid of residual vRNA2 were required to ensure that particles produced by vRNA1 and mutant trRNA2 were not contaminated with wild-type virions. Purification of vRNA1 by electrophoretic separation of viral RNAs on agarose gels failed to remove vRNA2 completely; this was evident by recovery of infectious virus from *Drosophila* cells inoculated with a sample of vRNA1 that had been purified by three cycles of electrophoresis (Table 2, line 1).

To eliminate the remaining traces of vRNA2, vRNA1 was selectively amplified in *Drosophila* cells as described previously (1) by taking advantage of its capability to replicate independently of vRNA2 (11). Cultures were inoculated with electrophoretically purified vRNA1 at about 1,000 molecules per cell, the highest input that permitted efficient elimination of residual vRNA2 through serial passages (Table 2). Progeny vRNA1 was purified by electrophoretic separation from total cellular RNA harvested at 18 h. After two serial amplifications, all traces of vRNA2 were eliminated, as indicated by the disappearance of viral infectivity from cells transfected with the amplified vRNA1 sample (Table 2, column 3). A third and fourth passage were performed to generate large vRNA1 stocks free of vRNA2. Passage four was used in all remaining experiments.

Yields of virus from cells transfected with RNA1 and RNA2 were determined by releasing virus from cells at 18 h, concentrating particles by pelleting, and analyzing the pellet by protein gel electrophoresis (Fig. 3). The highest yields were obtained when both RNAs were derived from virions (Fig. 3, lane 3). Substitution of viral RNA2 with in vitro-synthesized transcripts of RNA2 (Fig. 3, lanes 6 and 7) reduced the yields by about 60%. Attempts to replace both viral RNAs by transcripts further reduced the yields to unsatisfactory levels (data not shown). The yield could be improved by first amplifying RNA1 transcripts in *Drosophila* cells, a procedure which improves their replicative ability, probably by elimination of the four nonviral bases from their 3′ ends (1a); however, even with this strategy the amount of virus produced was barely detectable by protein electrophoresis (Fig. 3, lanes 4 and 5) and was too small for optical detection on sucrose density gradients (data not shown).

Site-directed mutagenesis of coat protein alpha and synthesis of cleavage-defective particles. The atomic structure of mature BBV particles suggests involvement of Asn-363 rather than Ala-364 in the cleavage mechanism (16a). With this in mind, Asn-363 was targeted for site-directed mutagenesis and replaced by aspartic acid, threonine, and alanine. The first two changes, Asn→Asp and Asn→Thr, required the substitution of one base at the nucleotide level, whereas the change Asn→Ala required a substitution of two bases.

Particles were synthesized in *Drosophila* cells by trans-

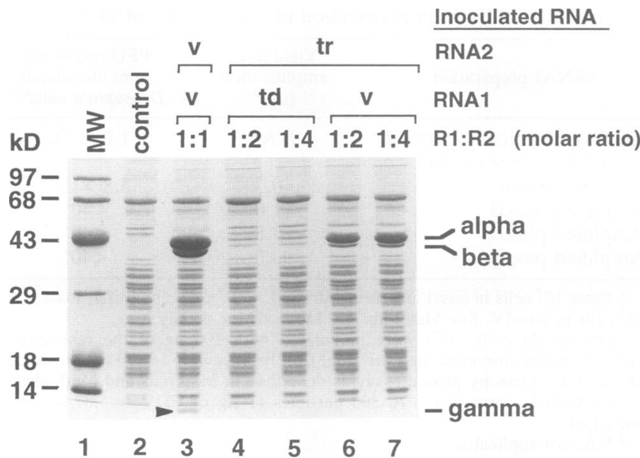

FIG. 3. Electropherogram showing synthesis of FHV particles in *Drosophila* cells transfected with different combinations of RNA1 and RNA2. RNA1 was either authentic vRNA (v) or derived from transcripts by amplification in *Drosophila* cells (td [transcript derived]). RNA2 was either authentic (v) or synthesized in vitro from a wild-type cDNA clone (tr). The numbers above lanes 3 to 7 indicate the molar ratios of RNA1 to RNA2 in the transfection mixture. Inoculated *Drosophila* cells (10^7) were lysed at 18 h by the addition of NP-40 to the culture medium. Nuclei were removed, and virus in the 2-ml extract was concentrated by pelleting through a 30% (wt/wt) sucrose cushion in a Beckman ultracentrifuge equipped with a TLA-100.3 rotor. Centrifugation was at 100,000 rpm for 13 min at 5°C, and then the pellet was resuspended in 30 μl of phosphate-buffered saline lacking calcium and magnesium. Aliquots of 6 μl each were subjected to electrophoresis, and proteins were stained with Coomassie brilliant blue. Lanes: 1, molecular mass (MW) markers; 2, high-speed pellet from cells transfected with water only; 3, high-speed pellet from cells transfected with 300 ng of authentic vRNA isolated from purified FHV containing 200 ng of vRNA1 and 100 ng of vRNA2; 4 to 7, high-speed pellet from cells transfected with 200 ng of indicated RNA1 and 200 ng of trRNA2 (ratio, 1:2 [lanes 4 and 6]) or 400 ng of trRNA2 (ratio, 1:4 [lanes 5 and 7]).

fection with equal amounts of purified vRNA1 and mutant trRNA2s. As a control, we also generated wild-type particles by using wild-type trRNA2. At 18 h, particles were purified from lysed cells by high-speed pelleting through a sucrose cushion followed by sucrose gradient sedimentation. No differences in the sedimentation rates of mutant and wild-type particles were observed (data not shown). Electrophoretic RNA analysis showed that virions contained the normal complement of RNA1 and RNA2 (data not shown). Particle yields were generally between 30 to 60 μg/10^8 cells except for the Asn→Asp mutant, which yielded only 5 to 10 μg/10^8 cells (Table 3).

Incubation of purified particles at room temperature for 24 h, followed by electrophoretic analysis on SDS-polyacrylamide gels, showed that all three mutants were cleavage defective (Fig. 4). Only wild-type particles displayed the typical coat protein cleavage pattern.

Infectivity of cleavage-defective particles. Specific infectivities of wild-type and mutant virions generated by RNA transfection were determined by plaque assay (Table 3, column 6). Specific infectivity of virions containing wild-type coat protein (2×10^8 PFU/μg) was indistinguishable from that of native virions. In contrast, specific infectivity of the Asp and Thr mutants decreased by factors of about 3,000 and 9,000, respectively, and the value for the Ala mutant

TABLE 3. Physicochemical properties and relative infectivities of gradient-purified wild-type and mutant FHV particles generated by RNA transfection of *Drosophila* cells

Wild-type or mutant virus	$OD_{260/280}$[a]	Yield (μg)[b]	RNA content	Cleavage of alpha protein[c]	Relative infectivity[d]
Wild type	1.60	66	Normal[e]	Yes (17% alpha; 83% beta)	1.0
Asn→Asp	1.43	6	ND[f]	No (100% alpha)	3.8×10^{-4}[g]
Asn→Thr	1.58	52	Normal	No (100% alpha)	1.0×10^{-4}[g]
Asn→Ala	1.54	30	Normal	No (100% alpha)	3.9×10^{-8}[g]

[a] $OD_{260/280}$, ratio of optical density at 260 nm to optical density at 280 nm ($OD_{260/280}$ of gradient-purified native virus, 1.56).

[b] Yield per 10^8 transfected cells; averages of two experiments.

[c] The extent of alpha chain cleavage was determined by densitometric analysis of the gel shown in Fig. 4 by using an Ultro Scan Laser Densitometer (Pharmacia LKB Biotechnology, Inc.).

[d] Relative to specific infectivity of wild-type virions (2×10^8 PFU/μg) generated by RNA transfection of *Drosophila* cells.

[e] RNA was extracted with phenol-chloroform from purified particles, and 2 μg was analyzed on a 1% agarose gel. No differences from the results with 2 μg of RNA extracted from native virions were observed.

[f] ND, not determined.

[g] Plaques were revertants to Asn (nine of nine); all Ala-to-Asn reversions require two base changes.

dropped to 3.9×10^{-8}. To test the possibility that the recovered infectivity was due to emergence of cleavage-competent revertants, virus was isolated from three independent plaques for each mutant. The plaque progeny were amplified, and RNA2 was sequenced through the cleavage site. In all cases, the nucleotide sequence at position 363 of alpha protein had reverted to that of wild type (data not shown). This suggested that the presence of Asp at position 363 was crucial for the cleavage mechanism and that cleavage-defective particles were not infectious.

DISCUSSION

The role of maturation cleavage in acquisition of nodaviral infectivity has been difficult to evaluate because of the inability to arrest spontaneous cleavage of coat precursor protein alpha. By taking advantage of the unusually long provirion half-life, we were able to obtain a preparation of virus particles enriched in provirions and found that maturation resulted in a five- to eightfold increase in specific plaque-forming infectivity. However, the experiment did not distinguish intrinsic infectivity of provirions from the background of infectivity due to mature or partially mature virions in the population. By replacing asparagine 363 at the cleavage site with aspartate, threonine, or alanine, we were able to construct cleavage-negative mutants with essentially no intrinsic infectivity as measured by plaque assay. There was no evidence for any assembly defect in mutant provirions. All mutants analyzed packaged a normal complement of RNA and their diameters were indistinguishable from those of wild-type virions as determined by sedimentation velocity.

Because native provirions attached almost as efficiently as mature virions to susceptible *Drosophila* cells, maturation cleavage is presumably required for some step between attachment and release of the genome into the cytosol. Nothing is yet known about steps involved in the uncoating of nodaviruses, but a few observations point to some intriguing similarities with picornaviruses. For example, FHV particles can be converted under certain conditions to 100S subparticles, which lack gamma chains (10). Loss of the internally located gamma chains from FHV is highly remi-

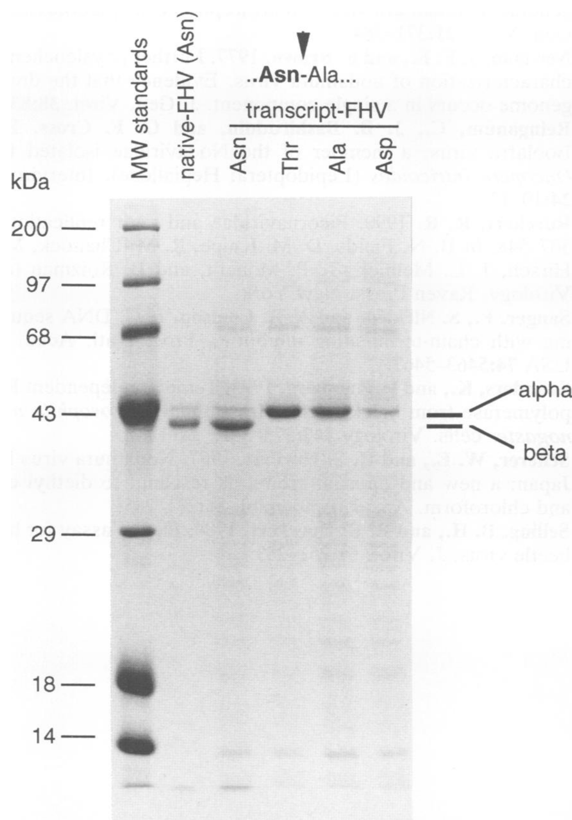

FIG. 4. Electropherogram showing cleavage phenotype of mutant virus particles constructed by replacing asparagine at the maturation cleavage site with three other amino acids. Virions, prepared by RNA transfection of *Drosophila* cells and purified as described in Materials and Methods, were incubated for 24 h at room temperature before electrophoretic analysis. Aliquots of 5 μg were loaded for each sample, and proteins were visualized by Coomassie brilliant blue. Sample loadings from left to right: molecular weight (MW) markers (lane 1), native FHV purified from FHV-infected *Drosophila* cells (lane 2), virus generated by inoculation of *Drosophila* cells with purified vRNA1 and trRNA2 (lanes 3 to 6). Lane 3, wild-type FHV; lane 4, Asn→Thr mutant; lane 5, Asn→Ala mutant; lane 6, Asn→Asp mutant. Protein gamma is not visible.

niscent of the release of internally located VP4 chains from picornaviruses, a process that generates RNA-containing A-particles, which are thought to be intermediates in the uncoating process (for a review, see reference 22). Studies of the attachment of cleavage-defective FHV provirions and the step at which infectivity is blocked are currently under way.

The time required for 50 to 75% of the maturation cleavages in provirions to occur is 4 to 8 h, considerably less than the 2 days required for plaque development on *Drosophila* cells. If provirions attach to cells with an efficiency similar to that of mature virions, why is their relative infectivity only 10 to 15% (Table 1)? One possibility is that provirions, which are more labile than mature virions, are degraded more rapidly during the infectious process. That infectivity of even mature virions is inefficient is suggested by the high particle-to-plaque forming ratio of 300:1, which is in the same range as that of picornaviruses. Alternatively, a lower plating efficiency of provirions may be caused by a significant delay in initiating infection. Plaque development of FHV on *Drosophila* cell monolayers has been shown to be highly sensitive to cell density (26). The delay, caused by the requirement for maturation cleavage before infection could be initiated, may have allowed the monolayer to reach inhibitory density before plaque formation could be completed.

Insights into the structural transitions of the viral capsid caused by cleavage of protein alpha require biophysical examination of the three-dimensional structure of the virus particle before and after maturation. The structure of the protein shell of mature FHV is already known in atomic detail (16a). We have shown here that it is possible to synthesize mutant provirions stable enough to conduct analogous studies of the structure of uncleaved virus particles. To obtain amounts suitable for crystallographic examination, we have already constructed recombinant baculoviruses expressing provirions that are stable during the 5-week time frame required to generate crystals needed for X-ray analysis (unpublished data). Hence, it is now feasible, in principle, to visualize in atomic detail the structural changes associated with acquisition of nodaviral infectivity.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI22813.

We thank Jack Johnson of Purdue University for valuable discussions leading to the choice of asparagine 363 as a target for mutagenesis.

REFERENCES

1. **Ball, L. A., J. M. Amann, and B. K. Garret.** 1992. Replication of nodamura virus after transfection of viral RNA into mammalian cells in culture. J. Virol. **66:**2326–2334.
1a. **Dasgupta, R.** Personal communication.
2. **Dasgupta, R., A. Ghosh, B. Dasmahapatra, L. A. Guarino, and P. Kaesberg.** 1984. Primary and secondary structure of black beetle virus RNA2, the genomic messenger for BBV coat protein. Nucleic Acids Res. **12:**7215–7223.
3. **Dasgupta, R., and J. Y. Sgro.** 1989. Nucleotide sequences of three nodavirus RNA2's: the messengers for their coat protein precursors. Nucleic Acids Res. **17:**7525–7526.
4. **Dasmahapatra, B., R. Dasgupta, A. Ghosh, and P. Kaesberg.** 1985. Structure of the black beetle virus genome and its functional implications. J. Mol. Biol. **182:**183–189.
5. **Dasmahapatra, B., R. Dasgupta, K. Saunders, B. Selling, T. Gallagher, and P. Kaesberg.** 1986. Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. Proc. Natl. Acad. Sci. USA **83:**63–66.
6. **Dearing, S. C., P. D. Scotti, P. J. Wigley, and S. D. Dhana.** 1980. A small RNA virus isolated from the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae). N. Z. J. Zool. **7:**267–269.
7. **Friesen, P. D., and R. R. Rueckert.** 1981. Synthesis of black beetle virus proteins in cultured *Drosophila* cells: differential expression of RNAs 1 and 2. J. Virol. **37:**876–886.
8. **Friesen, P. D., and R. R. Rueckert.** 1982. Black beetle virus: messenger for protein B is a subgenomic viral RNA. J. Virol. **42:**986–995.
9. **Friesen, P. D., and R. R. Rueckert.** 1984. Early and late functions in a bipartite RNA virus: evidence for translational control by competition between viral mRNA. J. Virol. **49:**116–124.
10. **Gallagher, T. M.** 1987. Ph.D. thesis. University of Wisconsin, Madison.
11. **Gallagher, T. M., P. D. Friesen, and R. R. Rueckert.** 1983. Autonomous replication and expression of RNA 1 from black beetle virus. J. Virol. **46:**481–489.
12. **Gallagher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral

insect ribovirus. J. Virol. **62**:3399–3406.

13. **Guarino, L. A., A. Ghosh, B. Dasmahapatra, R. Dasgupta, and P. Kaesberg.** 1984. Sequence of the black beetle virus subgenomic RNA and its location in the viral genome. Virology **139**:199–203.
14. **Guarino, L. A., and P. Kaesberg.** 1981. Isolation and characterization of an RNA-dependent RNA polymerase from black beetle virus-infected *Drosophila melanogaster* cells. J. Virol. **40**:379–386.
15. **Hendry, D. A.** 1991. Nodaviridae of invertebrates, p. 227–276. *In* E. Kurstak (ed.), Viruses of invertebrates. Marcel Dekker, Inc., New York.
16. **Hosur, M. V., T. Schmidt, R. C. Tucker, J. E. Johnson, T. M. Gallagher, B. H. Selling, and R. R. Rueckert.** 1987. Structure of an insect virus at 3.0 Å resolution. Proteins Struct. Funct. Genet. **2**:167–176.

16a. **Johnson, J. (Purdue University).** Personal communication.

17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Longworth, J. F., and R. D. Archibald.** 1975. A virus of black beetle, *Heteronychus arator* (F.) (Coleoptera: Scarabaeidae). N. Z. J. Zool. **2**:233–236.
19. **Newman, J. F. E., and F. Brown.** 1973. Evidence for a divided genome in nodamura virus, an arthropod-borne picornavirus. J. Gen. Virol. **21**:371–384.
20. **Newman, J. F. E., and F. Brown.** 1977. Further physicochemical characterization of nodamura virus. Evidence that the divided genome occurs in a single component. J. Gen. Virol. **38**:83–95.
21. **Reinganum, C., J. B. Bashiruddin, and G. F. Cross.** 1985. Boolarra virus: a member of the Nodaviridae isolated from *Oncopera intricoides* (Lepidoptera: Hepialidae). Intervirology **24**:10–17.
22. **Rueckert, R. R.** 1990. Picornaviridae and their replication, p. 507–548. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, New York.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
24. **Saunders, K., and P. Kaesberg.** 1995. Template-dependent RNA polymerase from black beetle virus-infected *Drosophila melanogaster* cells. Virology **147**:373–381.
25. **Scherer, W. E., and H. S. Hurlbut.** 1967. Nodamura virus from Japan: a new and unusual arbovirus resistant to diethyl ether and chloroform. Am. J. Epidemiol. **86**:271–285.
26. **Selling, B. H., and R. R. Rueckert.** 1984. Plaque assay for black beetle virus. J. Virol. **51**:251–253.