Capsid Protein Synthesis from Replicating RNA Directs Specific Packaging of the Genome of a Multipartite, Positive-Strand RNA Virus†

P. Arno Venter, Neel K. Krishna,‡ and Anette Schneemann*

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Received 12 October 2004/Accepted 18 January 2005

Flock house virus (FHV) is a bipartite, positive-strand RNA insect virus that encapsidates its two genomic RNAs in a single virion. It provides a convenient model system for studying the principles underlying the copackaging of multipartite viral RNA genomes. In this study, we used a baculovirus expression system to determine if the uncoupling of viral protein synthesis from RNA replication affected the packaging of FHV RNAs. We found that neither RNA1 (which encodes the viral replicase) nor RNA2 (which encodes the capsid protein) were packaged efficiently when capsid protein was supplied in trans from nonreplicating RNA. However, capsid protein synthesized in cis from replicating RNA2 packaged RNA2 efficiently in the presence and absence of RNA1. These results demonstrated that capsid protein translation from replicating RNA2 is required for specific packaging of the FHV genome. This type of coupling between genome replication and translation and RNA packaging has not been observed previously. We hypothesize that RNA2 replication and translation must be spatially coordinated in FHV-infected cells to facilitate retrieval of the viral RNAs for encapsidation by newly synthesized capsid protein. Spatial coordination of RNA and capsid protein synthesis may be key to specific genome packaging and assembly in other RNA viruses.

Flock house virus (FHV), a member of the Nodaviridae family, is a nonenveloped,icosahedral insect virus whose genome consists of two positive-strand RNA molecules (3, 28). The bipartite nature of the FHV genome organizes its nonstructural and structural genes onto RNA1 (3.1 kb) and RNA2 (1.4 kb), respectively. An open reading frame (ORF) that nearly spans the length of RNA1 encodes protein A (112 kDa), the RNA-dependent RNA polymerase which directs the replication of RNA1, RNA2, and a 387-nucleotide (nt) subgenomic RNA3 (7). RNA3 corresponds to the 3′ end of RNA1 and encodes two small proteins, B1 and B2 (10). No function has been ascribed to B1, while B2 functions as an inhibitor of RNA silencing (15). Interestingly, RNA3 is also required as a transactivator of RNA2 replication, but the mechanism by which this is achieved is not completely understood (6). RNA2 encodes the 43-kDa capsid precursor protein α, which is the only structural protein required for the assembly of FHV provirions (7, 9). Each provirion consists of 180 subunits of protein α, arranged with T=3 quasiequivalent symmetry, and the two genomic RNAs. Provirions are not infectious and undergo an autocatalytic maturation process in which protein α cleaves into proteins β (38 kDa) and γ (5 kDa). Both cleavage products remain associated with the matured, infectious virion (9, 26).

FHV RNA1 and RNA2 are packaged into a single virion but the mechanism by which FHV copackages its multipartite genome is still unknown (14). One hypothesis is that an interaction between RNA1 and RNA2 occurs prior to packaging, allowing the genome to be encapsidated as a complex. The absence of significant complementarity between RNA1 and RNA2, however, does not support this model. Moreover, it was recently shown that RNA1 can be packaged in the absence of RNA2 by a capsid protein variant that lacks N-terminal residues 2 through 31, further suggesting that a noncovalent complex between RNA1 and RNA2 is not formed prior to packaging (17). An alternate hypothesis is that the packaging of genomic RNAs occurs in a sequential manner, similar to what has been described for bacteriophage 46 (19). In this model, one genomic RNA strand would interact with a specific binding site on the capsid protein and the formation of this intermediate would allow for the binding of the second strand. No experimental evidence has been obtained to date to support this model.

We recently showed that FHV replication can be initiated in Sf21 cells from recombinant baculovirus vectors (13). Specifically, we demonstrated that the coinfection of Sf21 cells with baculoviruses containing the cDNA for RNA1 and RNA2 launches self-directed RNA replication that leads to the formation of progeny FHV particles. Many of these particles contain an accurate complement of the FHV genome. We have now used this system to uncouple the synthesis of protein A and capsid protein from the replication of RNA1 and RNA2 to investigate whether RNA1 can be packaged in the absence of RNA2 and vice versa. Our results show that capsid protein translated from a replication-competent RNA2 can package RNA2 in the absence of RNA1. However, capsid protein synthesized in trans from a nonreplicating template packages neither RNA1 nor RNA2, even when both RNAs are subject to replication by protein A. This indicates a previously unrecognized type of coupling of RNA replication and translation on one hand and RNA packaging on the other. We suspect that accurate assembly of infectious FHV particles normally occurs in cellular microenvironments where viral RNA and capsid...
protein synthesis are spatially coordinated. This constraint represents a new aspect of viral assembly that could be important for many other RNA viruses.

**MATERIALS AND METHODS**

Cells. *Spodoptera frugiperda* cells (line IPLB-SF21) (30) were propagated in TC100 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 27°C as described previously (24).

**Construction of recombinant transfer vectors for baculovirus expression.** The construction of DNA transfer vectors for the generation of recombinant baculoviruses AcR18 and AcR26 with the BacPAK expression system kit (BD Biosciences) was previously described (13). These vectors contain the cDNA of FHV RNA1 and RNA2, respectively, with the hepatitis delta virus (HDV) ribozyme sequence (22) located immediately adjacent to the 3' end of each cDNA insert.

To generate transfer vectors for AcR18 [-5'UTR], AcR2 [-3'UTR], and AcR [5'–3'UTR], the plasmid FHV[1,0] (2), in which the DNA of RNA1 is fused to the HDV ribozyme sequence at its 3' end, was utilized. For AcR18 [-5'UTR], a DNA fragment containing RNA1 and the HDV sequence was amplified by PCR with Phy-Turbo polynucleotide (Strategene) using primers that were designed to specifically delete the 3' untranslatable region (UTR) of RNA1. For AcR18 [5'–3'UTR] and AcR2 [5'–3'UTR] DNA fragments containing RNA1 were PCR amplified using primers that were designed to specifically delete the 3' UTR or both the 5' and 3' UTRs of FHV RNA1, respectively. The 3' UTR of RNA1 was defined as the 3'-terminal 71 nucleotides not encoding protein A. The forward primers used for the amplification of the previously described DNA fragments with 5' and/or 3' UTR deletions contained a 5'-terminal EcoRI restriction site, while the reverse primers corresponded to nucleotides 1088 through 1068 of FHV RNA1 and contained a BamHI restriction site. Following PCR, these DNA fragments were electrophoresed through an agarose gel in Tris-acetate-EDTA (TAE) buffer, purified using the Qiagen II purification kit (Qiagen), digested with EcoRI and XbaI, and ligated into the EcoRI-XbaI-digested gel-purified transfer vector pBacPAK9 (BD Biosciences).

To generate transfer vectors for AcR2 [-5'UTR], AcR2 [-3'UTR] and AcR2 [5'–3'UTR], the plasmid pBS(+) wt (26), containing the cDNA of RNA2, was utilized. For AcR2 [-5'UTR] DNA fragment containing RNA2 was PCR amplified using primers that were designed to specifically delete the 5' UTR of RNA2. For AcR2 [5'–3'UTR] and AcR2 [5'–3'UTR] DNA fragments containing RNA2 were PCR amplified using primers that were designed to specifically delete the 3' UTR or both the 5' and 3' UTRs of FHV RNA2, respectively. The forward and reverse primers used for the amplification of these DNA fragments contained 3'-terminal BamHI and 3'-terminal XbaI restriction sites, respectively. Following PCR, the DNA fragments were purified, digested with BamHI and XbaI, and ligated into the BamHI-XbaI-digested gel-purified pBacPAK9.

To generate transfer vectors for AcΔ31β and AcΔ31 [-3'UTR], plasmid pBSβ (3,4), which lacks the coding sequence for amino acids 2 through 31 of the FHV capsid protein, was utilized as a template for PCR. The forward primer corresponded to nucleotides 1 through 18 and had a BamHI site, while the reverse primer corresponded to nucleotides 1088 through 1068 of FHV RNA2. The resultant PCR product was purified and digested with BamHI and Hpal. The transfer vector for AcΔ31β was constructed by a three-way ligation reaction that involved (i) the BamHI-Hpal-digested PCR product, (ii) a Hpal-BamHI DNA fragment containing the remaining 3'-end-proximal 453 nt of FHV RNA2 flanked by the HDV sequence (obtained by digesting the AcR2 transfer vector with BamHI and Hpal and gel purifying a 542-bp DNA fragment), and (iii) BamHI-digested pBacPAK9. The transfer vector for AcΔ31 [-3'UTR] was constructed by a ligation reaction that involved (i) the BamHI-Hpal-digested PCR product and (ii) a Hpal-BamHI DNA fragment containing the remaining 300 nt of the FHV capsid protein reading frame followed by the pBacPAK9 vector sequence (obtained by digesting the AcR2 [-3'UTR] transfer vector with BamHI and Hpal and gel purifying a 5,836-bp DNA fragment).

The construction of a transfer vector for AcΔR28 (AUG→stop) involved two steps. First, the plasmid p2BSβ (AUG→stop) was used as a template for an inverse PCR (11) with primers that mutated the DNA sequence encoding the start (AUG) codon of the capsid protein ORF to a stop (UAG) codon. Second, the resultant plasmid, p2BSR28 (AUG→stop), was used as a template for a PCR with a forward primer that contained a BamHI site and a reverse primer corresponding to nucleotides 1088 through 1068 of FHV RNA2. The PCR product was digested with BamHI and Hpal and the transfer vector for AcR28 (AUG→stop) was constructed utilizing a similar three-way ligation strategy to the one described for AcΔ31β.

Following the transformation of competent DH5α cells, plasmid DNA was isolated from several clones and the presence and orientation of the inserted DNA were determined by diagnostic restriction endonuclease mapping. Upon restriction endonuclease screening, positive clones were sequenced to confirm that no errors were introduced by PCR.

**Generation of recombinant baculoviruses.** Recombinant baculoviruses were generated following the protocols of the manufacturer (BD Biosciences). In brief, each transfer vector was transfected into SF21 cells together with Bsu36I-linearized BacPAK6 baculoviral DNA (BD Biosciences), and cell supernatants were harvested 3 days posttransfection. Putative recombinant baculoviruses were plated on SF21 cells monolayers once on SF21 cell monolayers and amplified following confirmation of the expression of the inserted genes. The titers of the recombinant baculovirus stocks were determined by plaque assay and ranged from 0.1 × 10^5 to 2 × 10^6 PFU/ml.

**Infection of SF21 cells.** Monolayers consisting of 1.5 × 10^5 SF21 cells in six-well plates were infected at a multiplicity of infection (MOI) of either 5 or 10 PFU per cell by the addition of 0.5 ml sample containing recombinant baculovirus stocks. This was followed by a 1-h incubation at room temperature (with rocking), the removal of the unattached baculovirus, and the addition of 2 ml complete growth medium to each well. Incubation was continued at 27°C for 3 to 5 days without agitation.

**Preparation of RNA2 and RNA3 for liposome-mediated RNA transfection.** Capped RNA2 transcripts were synthesized by in vitro transcription from XbaI-linearized p2BS(+) wt as described previously (25). RNA3 was purified from SF21 cells infected with AcR18 as follows: total cellular RNA was purified from transfected SF21 cells using the RNeasy kit (QIAGEN), digested with DNase, and purified using the RNeasy kit (Qiagen). The RNA was electrophoresed through a nondenaturing 1% agarose gel in TAE and visualized with ethidium bromide present in the gel. A band of the expected size for RNA3 (387 nt) that was not present in uninfected SF21 cells was excised from the gel and purified using the RNeasy kit (QIagen).

**Infection/transfection of SF21 cells.** An infection/transfection protocol for the production of infectious FHV particles was described previously (13). In brief, monolayers consisting of 1.5 × 10^5 SF21 cells in six-well plates were infected with recombinant baculoviruses at an MOI of 5 for the expression of RNA1 or RNA1 UTR deletion mutants. This was followed by liposome-mediated transfection of 100 ng in vitro-synthesized capped RNA2 at 24 h postinfection. In some instances, approximately 100 ng gel-purified RNA3 was cotransfected with 100 ng RNA2 (the molar ratio of RNA3 to RNA2 was approximately 3.6:1). Incubation was continued at 27°C for 2 to 4 days posttransfection.

**Particulate purification of a virus-like particle.** Three days postinfection, baculovirus-infected or baculovirus-infected RNA-transfected SF21 cells in each well of a six-well plate were lysed by the addition of Nonidet P-40 to a final concentration of 0.5% (vol/vol). After an incubation of 10 min on ice, cell debris was pelleted at 16,000 × g for 10 min in a microfuge, and particles in the resultant supernatant were in turn pelleted through a 30% (wt/wt) sucrose cushion in 50 mM HEPES (pH 7)–0.1% bovine serum albumin–5 mM CaCl₂ at 243,000 × g for 45 min in a Beckman SW50.1 rotor. The pellet was resuspended in 50 µl of 50 mM HEPES, 0.5 mM EDTA, 600 mM NaCl, 1% sucrose, and 1% Triton X-100 in a microfuge for the exclusion of insoluble matter, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or immunoblotting.

**Purification of virus-like particles and infectious virions.** FHV particles were purified from SF21 cells 5 days postinfection by pelleting through a 30% (wt/wt) sucrose cushion followed by sedimentation through a 10 to 40% (wt/wt) sucrose gradient as described previously (13). The particles were harvested from the sucrose gradient by needle puncture or on an ISCO gradient fractionator at 0.75 ml/min and 0.5 min/fraction.

**RNA extraction from SF21 cells and purified particles.** Total cellular RNA was extracted from SF21 cells 3 days postinfection using the RNeasy kit (Qiagen), while RNA was extracted from purified FHV particles by means of phenol-chloroform extraction as described previously (25).

**Electrophoretic and Northern blot analyses of RNA.** Total cellular RNA was analyzed by denaturing agarose gel electrophoresis in the presence of formaldehyde, as described previously (27), with or without 2% Seakem LE high resolution gel (FMC) was used instead of a 1% agarose gel. RNA extracted from purified FHV particles was analyzed by electrophoresis through a nondenaturing 2% Seakem LE agarose gel in TAE buffer. In both cases, RNA was visualized with ethidium bromide. For Northern blot analysis, RNA was electrophoresed through a denaturing 2% Seakem LE agarose- formaldehyde gel and transferred to a nylon membrane as described previously (27). The probe used for hybridization was digoxigenin-UTP labeled and complementary to nucleotides 124 through 1400 of RNA2. The synthesis of this probe was previously described (13) and prehybridization, hybridization, and chemiluminescent detection were carried out according to the manufacturer’s protocols (Roche Molecular Biochemicals).

Reverse transcription-PCR (RT-PCR) analysis of RNA extracted from puri-
Stop was used as a template for in vitro transcription.

Electrophoresis and immunoblot analysis with rabbit anti-FHV serum were carried out as described previously (4).

Capped AUGs were sequenced. Capped AUG3 was cloned into the pCR4-TOP vector (Invitrogen) and sequenced. As a control, cDNA fragments were expected to produce functional RNA polymerase. Figure 1A shows a schematic representation of the baculovirus deletion mutants AcR16[−5′UTR], AcR1[−3′UTR], and AcR1[−3′3′UTR].

To determine if the elimination of the UTRs inhibited RNA1 replication, Sf21 cells were infected with each of the AcR1 deletion mutants described above, and total cellular RNA was extracted 3 days after infection. Electrophoretic analysis of the RNA samples demonstrated that, relative to a control of AcR16-infected cells, AcR16[−5′UTR]-infected cells contained only faintly detectable levels of RNA1 and reduced levels of the subgenomic RNA3 (Fig. 1B, lane 3). This result was in line with previous observations that in the absence of the 5′ UTR, protein A does not efficiently replicate RNA1 (2). While the absence of the 5′ UTR was not expected to affect the synthesis of RNA3 (5), the overall reduced level of RNA1 resulted in a concomitant decrease of the subgenomic RNA. As expected, neither RNA1 nor RNA3 was detected in AcR1[-3′UTR]- and AcR1[−5′3′UTR]-infected cells (Fig. 1B, lanes 4 and 5). Analysis of cell lysates by protein gel electrophoresis showed that the levels of protein A were similar regardless of the absence or presence of the UTRs (data not shown), indicating that amplification of RNA1 by replication did not lead to a corresponding increase in protein production. This was reminiscent of the situation for FHV-infected Drosophila cells, which contain very high levels of RNA1 but only low levels of protein A (7).

To confirm that all three UTR deletion mutant types, in particular the 3′ UTR deletion mutants, directed the synthesis of functional protein A, Sf21 cells were infected with one of the three recombinant baculoviruses and transfected 24 h later with a mixture of RNA2 and RNA3. The rationale was that if functional RNA polymerase were present, then it would rep-

**RESULTS**

Deletion of the RNA1 3′ UTR effectively disables RNA1 replication but not FHV replicase synthesis. We recently demonstrated that FHV RNA1 replicates to high levels in Sf21 cells when it is launched from the recombinant baculovirus AcR16 (13). AcR16 contains the cDNA of RNA1 directly followed by the hepatitis delta virus ribozyme sequence (Fig. 1A) (13).

After the transcription of the RNA1 gene from the baculovirus DNA and self-cleavage of the HDV sequence, an RNA1 product that contains an authentic 3′ end and only a few heterologous nucleotides at the 5′ end is obtained. Translation of this RNA1 yields protein A, which in turn amplifies the RNA1 message and also produces the subgenomic RNA3 (Fig. 1B, lane 2). To produce protein A in the absence of RNA1 replication, we generated three new recombinant baculoviruses in which the 5′ UTR, 3′ UTR, together with the HDV site, or all were deleted. The rationale was based on previous studies that showed that the deletion of the 5′ UTR severely reduces the efficiency of RNA1 replication, whereas deletion of the 3′ UTR completely abolishes it (1, 2, 16). All UTR deletion mutants carried an intact ORF for protein A and were expected to produce functional RNA polymerase.

![Diagram](http://jvi.asm.org/)
aggressive. Since the maturation cleavage is assembly dependent (9). The presence of transfected RNA3 was expected, since AcR1 that high levels of capsid protein were synthesized in the absence of RNA3. Cell lysates were prepared 3 days after transfection and subjected to immunoblot analysis using an FHV antiserum.

FIG. 2. Immunoblot of Sf21 cell lysates infected with various AcR1 constructs and transfected with RNA2 in the presence or absence of RNA3. Sf21 cells were infected with the indicated baculoviruses at an MOI of 5 and transfected 24 h later with RNA2 in the presence (+) or absence (−) of RNA3 (the molar ratio of RNA2 to RNA3 was approximately 1:3.6). Cell lysates were prepared 3 days after transfection and subjected to immunoblot analysis using an FHV antiserum.

licate and thereby amplify RNA2 and RNA3 in trans as we had shown previously for AcR1 (13). This, in turn, would result in the synthesis of capsid protein and assembly of virus particles, which could be detected by immunoblot analysis. In a separate set of experiments, baculovirus-infected Sf21 cells were transfected with RNA2 but not RNA3. Recall that the replication of RNA2 requires RNA3 as a transactivator (6). Since the detection of capsid protein by immunoblot analysis is possible only after the amplification of RNA2 by replication (unpublished results), this set of experiments served as a convenient negative control.

As shown in Fig. 2 (lanes 1 and 2), cells infected with AcR16[−5’UTR] and transfected with RNA2 in either the presence or absence of RNA3 contained abundant amounts of capsid protein α and the cleavage product β. The presence of protein β indicated that capsid protein had formed particles, since the maturation cleavage is assembly dependent (9). The other cleavage product, protein γ, is too small (5 kDa) to be visualized on standard Laemmli polyacrylamide gels. The fact that high levels of capsid protein were synthesized in the absence of transfected RNA3 was expected, since AcR16[−5’UTR] gave rise to sufficient amounts of RNA3 itself (Fig. 1B, lane 3). In contrast, Sf21 cells that were infected with one of the 3’ UTR deletion constructs produced capsid proteins α and β only when cotransfected with RNA2 and RNA3 (Fig. 2, lanes 3 to 6). The levels of coat protein in these cells differed from experiment to experiment and are lower in the particular experiment shown here, but this was not consistently observed. Taken together, the results provided indirect confirmation that the cells contained functional protein A.

RNA2 is efficiently packaged in the absence of RNA1 replication. The infection/transfection system described above provided an opportunity to determine whether capsid protein could package RNA2 in the absence of replicating RNA1. This allowed us to address the question of whether the viral RNAs were packaged independently of each other. To test this, FHV particles were sucrose gradient purified from cells supporting the replication of RNA2 but not RNA1 (infected with AcR1[−3’UTR] or AcR1[−5’3’UTR] and transfected with RNA2 and RNA3) and, as a control, from cells supporting replication of both RNA1 and RNA2 (infected with AcR16 or AcR16[−5’UTR] and transfected with RNA2). RNA was then extracted from the particles and analyzed on a nondenaturing agarose gel. RNA1 and RNA2 were packaged by FHV capsid protein in cells that supported the replication of both RNAs (Fig. 3, lanes 1 and 2). The population of purified particles also contained random cellular RNA, as we observed previously (13).

The slight decrease in the ratio of RNA1 to RNA2 for particles purified from AcR16[−5’UTR]-infected cells (Fig. 3, lane 2) compared to AcR16-infected cells (Fig. 3, lane 1) was probably the result of the proportionately lower levels of RNA1 available for packaging in AcR16[−5’UTR]-infected cells (Fig. 1B, lane 3). In contrast, considerable quantities of RNA2 (and cellular RNA), but no RNA1, were detected in particles isolated from cells supporting the replication of RNA2 but not RNA1 (Fig. 3, lanes 3 and 4). This result demonstrated that RNA2 could be packaged in the absence of RNA1 replication.

Deletion of the RNA2 3’ UTR effectively disables RNA2 replication. We next wanted to test the reverse scenario: could RNA1 be packaged in the absence of RNA2 replication? This required an expression system in which replication-competent RNA1 was coexpressed with replication-incompetent RNA2 (for the synthesis of FHV capsid protein). Accordingly, the 5’ UTR and/or 3’ UTR sequences present in AcR2 were deleted with a strategy analogous to that described above for RNA1 except that the HDV site was not retained at the 3’ end of the 5’ UTR deletion construct.

Figure 4A shows a schematic representation of recombinant baculoviruses for the expression of RNA2 with a 5’ UTR deletion (AcR2[−5’UTR]), a 3’ UTR deletion (AcR2[−3’UTR]), and
Polyhedrin promoter

Acr2β

RNA2

23 (AUG) 1244 (UAG)

RNA3

AcR2[−5′UTR]

AcR2[−3′UTR]

AcR2[−5′−3′UTR]

HCV ribozyme sequence

1400

B

AcR1 + AcR2[−5′UTR]

AcR1 + AcR2[−3′UTR]

AcR1 + AcR2[−5′−3′UTR]

AcR1 + AcR2[−5′−3′UTR]

RNA1

RNA2

RNA3

FIG. 4. Effect of deletion of the RNA2 5′ and/or 3′ UTR on RNA2 replication. (A) Schematic representation of recombinant baculoviruses for the synthesis of RNA2 and RNA2 UTR deletion mutants. AcR2β, a previously described construct (13), carries the complete cDNA sequence of RNA2, while other constructs carry deletions in the 5′ and/or 3′ UTRs. The first and last nucleotides of FHV RNA2, as well as the positions of start (AUG) and stop (UAG) codons of the FHV capsid protein ORF, in AcR2β are indicated. (B) Electrophoretic analysis (top panel) and Northern blot analysis (bottom panel) of total cellular RNA isolated from SF21 cells 3 days after infection at an MOI of 10 with AcR1β and indicated AcR2 constructs. Aliquots of either 3 µg (top panel) or 100 ng (bottom panel) RNA were run on a denaturing agarose gel. Northern blot analysis was performed with a negative-sense RNA2 probe. RNA isolated from authentic FHV particles (vRNA) and gel-purified RNA3 were run for comparison in lanes 6 and 7, respectively. The molecular sizes of RNA markers (in nucleotides) are indicated to the left.

the deletion of both UTRs (Acr2[−5′−3′UTR]). Immunoblot analysis of lysates from SF21 cells individually infected with these constructs showed no difference in the expression levels of the coat protein (data not shown). To determine if the UTR deletions inhibited RNA2 replication, SF21 cells were coinfected with AcR1β and AcR2β, AcR2[−5′UTR], AcR2[−3′UTR], or AcR2[−5′−3′UTR]. Electrophoretic analysis of total cellular RNA 3 days after infection showed that RNA1 was replicated with equal efficiency in all cases (Fig. 4B, top panel). A band comigrating with wild-type RNA2 was detected in cells infected with AcR2β and AcR2[−5′UTR] but not in those infected with the 3′ UTR deletion constructs (Fig. 4B, top panel). Northern blot analysis with a negative-strand RNA2 probe confirmed that this band represented RNA2. Since this clone did not carry the HDV site, its 3′ end was not identical to that of native RNA2. Therefore, it was expected that the FHV polymerase would not recognize it as a template for RNA replication (1). The fact that it did suggested that protein A is capable of recognizing replication elements within the 3′ UTR of RNA2 irrespective of the presence of additional heterologous nucleotides at the 3′ end.

Several additional bands of higher molecular mass were also detected and most probably represented primary RNA2 transcripts that had terminated at one of several polyhedrin gene termination signals and that had not cleaved at the HDV ribozyme site as observed previously (13). Such primary RNA transcripts could also be detected for the 3′ UTR deletion mutants upon longer exposure of the blot (not shown).

It has been demonstrated previously that not only does RNA3 transactivate RNA2 replication but its synthesis is repressed at the onset of RNA2 replication (8, 32). Therefore, additional proof that RNA2 was not replicated in cells infected with AcR2β[−3′UTR] or AcR2β[−5′−3′UTR] was manifested in the detection of higher levels of RNA3 in these cells than in AcR2β-infected cells (Fig. 4B, top panel, compare lanes 4 and 5 to lane 2). Taken together, these results demonstrated that RNA2 replication, like that of RNA1, was slightly inhibited by the 5′ UTR deletion but completely inhibited by the 3′ UTR deletion. As shown below, FHV capsid protein was, however, expressed from these nonreplicating RNA2 templates.

RNA1 is not efficiently packaged in the absence of RNA2 replication. To determine if RNA1 could be packaged in the absence of replicating RNA2, FHV particles were purified from SF21 cells supporting the replication of RNA1 in the absence of RNA2 replication (coinfected with AcR1β and either AcR2β[−3′UTR] or AcR2β[−5′−3′UTR]) and, as a control, from cells supporting the replication of both RNA1 and RNA2 (coinfected with AcR1β and either AcR2β or AcR2β[−5′−3′UTR]). As expected, both RNA1 and RNA2 were packaged by FHV capsid protein, together with random cellular RNA, in cells that supported the replication of RNA1 and RNA2 (Fig. 5, lanes 2 and 3). Surprisingly, the examination of the RNA packaged into particles from cells that supported only the replication of RNA1 revealed the presence of random cellular RNA only (Fig. 5, lanes 4 and 5). No RNA1 was detectable, despite the fact that high levels of this RNA were available for packaging in the cells in which these particles were assembled (Fig. 4B, top panel, lanes 4 and 5).
A capsid protein deletion mutant lacking residues 2 to 31 (Δ31) cannot package RNA1 when it is synthesized from non-replicating mRNA. There were two possible explanations for the inability of capsid protein to package RNA1 in the absence of RNA2 replication. First, it was possible that the packaging of RNA1 was dependent on the packaging of RNA2, i.e., that the packaging of FHV genomic RNAs occurs in a sequential manner, starting with RNA2. Second, it was possible that RNA1 was not packaged efficiently because it was somehow dependent on the replication of RNA2. To distinguish between these two possibilities, we decided to take advantage of a capsid protein mutant (Δ31) that lacks N-terminal residues 2 to 31. This mutant is able to package RNA1 independently of RNA2 in FHV-infected Drosophila cells (17). Thus, we reasoned that this mutant would package RNA1 in the absence of replicating RNA2 unless RNA2 replication was key to packaging FHV RNAs.

Two baculovirus expression vectors were constructed for the synthesis of replicating and nonreplicating Δ31 RNA2. The baculoviruses for the synthesis of replicating Δ31 RNA2 (AcΔ31) and nonreplicating Δ31 RNA2 (AcΔ31[−3’UTR]) were identical to AcR2 and AcR2[−3’UTR], respectively, with the exception that sequences encoding residues 2 to 31 of protein had been removed (compare Fig. 4A and Fig. 6A). Analogous to our previous observations with constructs expressing wt capsid protein, Sf21 cells coinfected with AcR1 and either AcΔ31 or AcΔ31[−3’UTR] contained equivalent amounts of RNA1 (Fig. 6B, lanes 1 and 2). A band representing Δ31 RNA2 transcripts, however, could not be clearly distinguished from other bands in the total RNA sample. We therefore used Northern blot analysis to confirm that Δ31 RNA2 replication products were present at high levels in AcR1/AcΔ31-infected cells (Fig. 6C, lane 1) and were absent in AcR1/AcΔ31[−3’UTR]-infected cells (Fig. 6C, lane 2). Higher-molecular-weight primary Δ31 RNA2 transcripts did become detectable upon longer exposure of this blot (not shown).
The results obtained so far pointed to a link between RNA2 replication and FHV RNA packaging. However, it was unclear whether replication of RNA2 per se was sufficient for packaging of the FHV genome or whether there was an additional link between replication of RNA2 and subsequent translation into capsid protein. To address this issue, we generated a recombinant baculovirus that encoded an RNA2 replicon with a closed capsid protein reading frame. This baculovirus, AcR26[AUG→Stop], was identical to AcR26 (Fig. 4A) with the exception that the start (AUG) codon of the capsid protein ORF was mutated to a stop (UAG) codon. To confirm that capsid protein synthesis but not RNA2 replication was disabled, Sf21 cells were infected with AcR26[AUG→Stop] in the presence or absence of AcR16. The detection of an RNA species with a similar molecular weight to RNA2 for AcR16/AcR26[AUG→Stop]-infected cells (Fig. 8A, lane 2) but not for AcR26[AUG→Stop]-infected cells (Fig. 8A, lane 1) demonstrated that AUG→Stop RNA2 was replicated to high levels in Sf21 cells supporting FHV RNA replication. As expected, FHV capsid protein was undetectable in these cells as demonstrated by immunoblot analysis (data not shown).

In the following analysis, Sf21 cells were coinfected with baculoviruses AcR28[AUG→Stop], AcR16, and AcR26 to confirm that AUG→Stop RNA2 could be packaged by capsid protein that was synthesized in trans from replicating wt RNA2. In the more critical experiment, Sf21 cells were infected with AcR26[AUG→Stop], AcR18, and AcR22[–5′3′UTR]. The latter experiment would reveal whether there was any difference in FHV RNA packaging when capsid protein was synthesized in trans from a nonreplicating template. FHV particles were purified from the infected cells, and RNA was extracted and subjected to agarose gel electrophoresis. Northern blot analysis with probes for RNA2 and RT-PCR were used to examine the nature of the packaged RNA.

As shown in Fig. 8B, particles purified from cells that were coinfected with viruses that included AcR26 contained both RNA1 and RNA2 (lane 1). To determine whether the packaged RNA2 included both wt and AUG→Stop products, it was amplified by RT-PCR with primers specific for RNA2 and the resulting products ligated into DNA vectors. Sequence analysis of 10 independent clones showed that the ratio of plasmids with an inserted AUG→Stop sequence to plasmids with an inserted wild-type RNA2 sequence was 1.5:1. This result proved that (i) AUG→Stop RNA2 could be packaged by capsid protein synthesized in trans from a replicating wt RNA2 and (ii) that the packaging efficiencies of the two RNAs were similar if not identical. We also performed a control experiment in which an equimolar mixture of in vitro-synthesized wt RNA2 and AUG→Stop RNA2 transcripts was subjected to RT-PCR, cloning, and sequencing. The same ratio (1.5:1) for wt RNA2 to AUG→Stop RNA2 was obtained.

Interestingly, particles purified from cells infected with the baculovirus that produced capsid protein from a nonreplicating template contained predominantly random cellular RNA (Fig. 8B, top panel, lane 2). This was despite the fact that high levels of RNA1 and AUG→Stop RNA2 were available for packaging in these cells (Fig. 8A, lane 3). A faint band that appeared to comigrate with RNA2 was visible in the collection of packaged RNAs, but it did not hybridize with an RNA2-specific probe in the Northern blot analysis (Fig. 8B, bottom panel, lane 2). Taken together, these results showed conclu-
sively that packaging of the FHV genomic RNAs requires capsid protein synthesized from a replicating RNA2.

**DISCUSSION**

This study describes the application of a baculovirus expression system for the synthesis of functional FHV proteins from either replicating or nonreplicating genomic RNA templates. This system was reliant on the removal of the 3′ UTRs from RNA1 and RNA2, which was shown to result in the inhibition of RNA replication with retention of protein A and capsid protein synthesis, respectively. The ability to effectively uncouple viral protein synthesis from RNA replication in this baculovirus expression system was used to determine if RNA2 could be packaged in the absence of RNA1 and vice versa. Examination of the RNA content of particles assembled in the presence of nonreplicating RNA1 and replicating RNA2 demonstrated that RNA2 was packaged efficiently in the absence of RNA1 replication. The reverse was, however, not true, as replicating RNA1 was not readily detected in particles that were assembled in the absence of RNA2 replication. We initially interpreted the results to indicate that the packaging of the two RNAs is sequential and that it must begin with RNA2. However, subsequent experiments revealed that the inability of the capsid protein to package RNA1 was due to the fact that the capsid protein had been produced from a nonreplicating template. This became obvious when neither replicating RNA1 nor replicating RNA2 were packaged by capsid protein translated from a primary baculovirus transcript. The RNAs were packaged, however, when capsid protein was synthesized from replicating wild-type RNA2. This result suggested some sort of coupling of replication translation of RNA2 on one hand and FHV RNA packaging on the other.

Coupling of RNA replication and packaging has previously been observed for poliovirus (20). In this case, nonreplicating poliovirus subgenomic RNAs were not trans-encapsidated by capsid protein generated from a replicating poliovirus genome. This prompted the investigators to propose that the encapsidation of poliovirus RNAs requires a direct physical interaction between the replication complexes and the assembling capsid protein. Such a direct link between replication complexes and assembling capsid protein is unlikely for FHV because pulse-chase experiments have shown that the genomic RNAs are packaged long after they have been synthesized by the FHV polymerase (9). Also, in FHV, the replication of the viral RNAs is not sufficient for packaging, based on our finding that replicating RNA1 and AUG→Stop RNA2 were not packaged by capsid protein synthesized in trans from a nonreplicating template. At the same time, the possibility of a linkage in cis between RNA packaging and translation could also be excluded because nontranslating AUG→Stop RNA2 was efficiently packaged by capsid protein made in trans from wild-type, replicating RNA2.

Our results can be rationalized by hypothesizing that RNA2 replication is required for the packaging of FHV RNAs because it guarantees that capsid protein is synthesized in a cellular location that permits immediate access to progeny RNA1 and RNA2. More specifically, we suspect that the trans-

![Figure 8](http://jvi.asm.org/)
Although there is no experimental evidence that this coordination is crucial for the accurate formation of replication, translation, and assembly are spatially coordinated and of cellular microdomains or compartments in which replication occurs near or at the site of replication itself, thereby ensuring that capsid protein does not have to traffic through the cytosol in order to retrieve the viral RNAs for assembly. This model, illustrated schematically in Fig. 9, explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly. Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.

Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.

Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.

Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.

Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.

Alternatively, it is possible that capsid protein, now translated from the replicated progeny RNA, was in the appropriate location for selecting the viral genome.

The scenario that we imagine for specific packaging of FHV RNAs also explains why the population of FHV particles produced in S21 cells from baculovirus vectors yielding replicating RNA1 and RNA2 always included particles that contain cellular RNA. This is because only a fraction of the primary transcripts are immediately translated, and the resulting capsid protein invariably packages cellular RNA as we showed many years ago (24). What this implies is that capsid proteins translated from replicating as opposed to nonreplicating RNAs assemble into particles that segregate into distinct populations and that only the population derived from replicating RNA contains the FHV genome. We are currently in the process of confirming this by using capsid proteins that display differential epitopes depending on what kind of template they are derived from.

The hypothesis that capsid protein must be synthesized near the site of RNA replication to ensure specific packaging of the FHV genome calls into question the existence of bona fide encapsidation signals on the viral RNAs. It could be argued that in the type of situation that we propose, RNA packaging relocation of the replication complexes to the site of assembly.
sidation signal has already been identified for a defective interfering RNA derived from RNA2 (31). When this signal is deleted, the defective interfering RNA2 is no longer packaged. Secondly, the principle of mass action does not satisfactorily explain the high fidelity with which RNA1 and RNA2 are copackaged, and thirdly, it does not explain why the subgenomic RNA3 is not packaged. Thus, further experiments are required to elucidate the molecular mechanism that ensures the copackaging of FHV RNA1 and RNA2 into progeny virions.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (GM53491 and C02181) and a National Institutes of Health fellowship award (AI10262) to N.K.K.

We thank J. K. Lannman for helpful suggestions during the preparation of the manuscript.

REFERENCES