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Evidence that the packaging signal for nodaviral RNA2 is a bulged stem–loop

(defective-interfering RNA/bipartite genome/RNA packaging)

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ABSTRACT **Flock house virus is an insect virus belonging to the family Nodaviridae; members of this family are characterized by a small bipartite positive-stranded RNA genome. The larger genomic segment, RNA1, encodes viral replication proteins, whereas the smaller one, RNA2, encodes coat protein. Both RNAs are packaged in a single particle. A defective-interfering RNA (DI-634), isolated from a line of *Drosophila* cells persistently infected with Flock house virus, was used to show that a 32-base region of RNA2 (bases 186–217) is required for packaging into virions. RNA folding analysis predicted that this region forms a stem–loop structure with a 5-base loop and a 13-base-pair bulged stem.**

Flock house virus (FHV) (1) is a small nonenveloped insect virus belonging to the family Nodaviridae (for review see ref. 2). Its RNA genome, among the smallest of any known animal virus, consists of only 4506 bases split between two single-stranded, messenger-sense RNA molecules, RNA1 and RNA2 (3–7); both are packaged in a single virion (8). Genomic RNA1, 3106 bases, contains all of the viral genes required for replication (9); it is the messenger for protein A (112 kDa) (5, 10, 11) and encodes the second protein B (10 kDa) through a subgenomic RNA3 (389 bases), which is not packaged (5, 12, 13). Genomic RNA2, 1400 bases, encodes virion coat protein precursor, α (47 kDa), which is cleaved into a 43-kDa (β) and a 4-kDa (γ) fragment after assembly into provirions (5, 14–16). RNA1 and RNA2 are required for viral infectivity (9). The mechanism by which RNA1 and RNA2, but not subgenomic RNA3, are selected for packaging into virions has been unclear.

FHV infects *Drosophila melanogaster* cells and normally causes lysis (17). However, about 1% of cells survive each infection cycle and become resistant to further infection (18). Such persistently infected cells synthesize, in addition to the genomic RNAs 1 and 2, defective RNAs, some of which are packaged into virions. Here we describe the use of one such molecularly cloned mutant, DI-634 with large deletions in RNA2, to identify a specific region of RNA required for packaging.

MATERIALS AND METHODS

Synthesis of Full-Length DNA Copy of DI-634, a Defective-Interfering (DI) RNA Derived from FHV RNA2. Gel-purified DI-634 was reverse-transcribed into cDNA and converted to double-stranded form with reverse transcriptase (19). The double-stranded DNA was cloned into transcription vector pBluescribe M13 (+) (Stratagene) following the sequence of T3 promoter. To generate transcripts with 5′ ends identical to those in authentic DI-634, the clone was further modified by removing the nonviral bases located between the transcription initiation site of T3 promoter and the cloned DI DNA through oligonucleotide-directed mutagenesis. Such RNA transcripts, however, still had four extra nonviral bases at the 3′ end (4).

***In Vitro* Transcription of Cloned FHV DNA.** Selected DNA clones were cleaved with the restriction enzyme *Xba* I, and the resulting linear DNA templates (0.03 mg/ml) were transcribed with T3 RNA polymerase as described by Konarska *et al.* (20). One-half millimolar guanosine(5′)triphospho(5′)guanosine [G(5′)ppp(5′)G] was included in the reaction mixture to provide capped transcripts. The ^{32}P-labeled negative-sense RNA2, used as the probe in Northern blot hybridization, was transcribed *in vitro* from *Pst* I-linearized RNA2 clone in pSP65 (Promega) using SP6 RNA polymerase. About 20 μCi of [α-^{32}P]CTP was included in a 25-μl reaction volume (1 Ci = 37 GBq).

Transfection of *Drosophila* Cells. A monolayer of vigorously growing *D. melanogaster* cells (17, 21) (8×10^6 cells) was used for a typical transfection reaction. RNA mixture (30 μl) containing 250 ng of purified FHV RNA1, 20–60 ng of RNA2, and 100 ng of DI transcripts was mixed with an equal volume of Lipofectin (BRL) and incubated at room temperature for 15 min. The prepared RNA/Lipofectin mixture was combined with 1 ml of serum-free Schneider culture medium (22) and loaded onto the *Drosophila* cell monolayer already washed three times with serum-free Schneider medium. The cells were incubated at 26°C for 1 hr followed by addition of 5 ml of Schneider culture medium supplemented with 15% fetal bovine serum. The transfected cells were grown at 26°C for about 40 hr before harvesting virions. For transfection of fewer cells, the amount of viral RNA used was reduced accordingly.

Purification of Virus Particles and Extraction of Virion RNA. About 40 hr after transfection, cells were lysed in the spent medium supplemented with 0.5% Nonidet P-40 and 0.1% 2-mercaptoethanol. Virus particles were purified as described (5). To recover virion RNA, gradient fractions containing virus were made 0.2 M in NaCl and 1% in sodium dodecyl sulfate (SDS) and then extracted twice with phenol and once with chloroform. After ethanol precipitation, the RNA pellet was washed with 70% ethanol and dissolved in distilled water. Ribonuclease A (10 μg/ml), sometimes included at the lysis step in the virus purification procedure, had no effect on identity of the RNA recovered from purified virions.

Radiolabeling and Extraction of Intracellular RNA. Intracellular RNA of infected cells was labeled by incorporation of [5,6-^{3}H]uridine (100 μCi/ml) (New England Nuclear) in the presence of actinomycin D (12 μg/ml) (Sigma) for a 2-hr period. To isolate total RNA, the cells were washed once with ice-cold TNE buffer (0.03 M Tris·HCl, pH 8.0/0.1 M

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Abbreviations: FHV, Flock house virus: DI, defective-interfering; nt, nucleotide(s); BBV, Black beetle virus; BOV, Boolarra virus; NOV, Nodamura virus.

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NaCl/25 mM EDTA) and then lysed with 0.4 ml of TNE buffer/0.1% SDS solution. The cell lysate was immediately extracted twice with phenol/chloroform and RNA was precipitated with ethanol.

RNA Analysis. [^{3}H]Uridine-labeled RNA was subjected to electrophoresis on a 10-cm 1.4% agarose gel (buffer was 89 mM Tris/89 mM borate/1 mM EDTA, pH 8.3). En^{3}Hance (New England Nuclear) was used in fluorography.

Unlabeled virion RNA recovered from purified virus particles was analyzed using Northern blot hybridization (23). Briefly, virion RNA (5–20 ng) fractionated on a 10-cm 1% agarose gel was transferred to a Zeta-Probe blotting filter (Bio-Rad) and hybridized with ^{32}P-labeled negative-sense RNA2 probe for about 20 hr at 65°C in the blotting buffer containing 50% formamide, 0.8 M NaCl, 0.5% SDS, 10× Denhardt's solution, and 0.25 mg of denatured salmon sperm DNA per ml. Signals were detected by autoradiography.

RESULTS

Origin and Structure of the DI RNA, DI-634, Used in Encapsidation Study. Virions purified from several lines of *Drosophila* cells persistently infected with FHV were found to package defective FHV RNAs (18). A number of such RNA molecules have been cloned and sequence analysis showed that they were derived from either RNA1 or RNA2. DI-634, the one described in this report, strongly inhibited replication of RNA2 when cotransfected with an equivalent concentration of virion RNAs 1 and 2 into *Drosophila* cells (data not shown). Sequence analysis of this DI-RNA revealed 634 bases representing three segments (I, II, and III) of RNA2 (Fig. 1). These three regions correspond to the first 249 bases at the 5′ end (segment I; bases 1–249), a middle region from base 517 to base 728 (segment II; bases 517–728), and the last 173 bases at the 3′ end (segment III; bases 1228–1400) of RNA2 sequence and they are arranged in the same order as in RNA2.

Regions Required for Replication of DI-634. The ability of DI-634 to be replicated was measured by cotransfecting transcripts from its cDNA clone into *Drosophila* cells together with purified viral RNA1, which encodes the polymerase needed for viral RNA replication (9). At 20 hr postinfection, newly synthesized viral RNA was radiolabeled with [^{3}H]uridine; the RNA was then extracted, separated electrophoretically, and detected by fluorography. Such experiments showed that DI-634 required three cis-acting regions for efficient replication; the first, about 124 bases in segment I; the entire segment II; and the last, about 65 bases in segment III (Fig. 1); the regions shown in black in Fig. 1 could be removed without affecting its replication (data not shown).

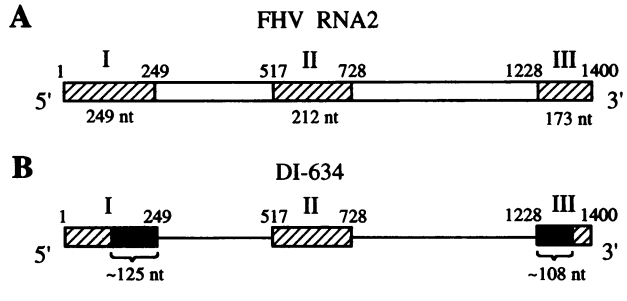

FIG. 1. Structures of RNA2 (1400 bases, *A*), which encodes the coat protein of FHV, and DI-634 (634 bases, *B*), a spontaneous deletion product that is efficiently replicated and packaged into virions and must therefore contain all of the sequence elements required for those functions. Shaded segments I, II, and III (*A*) represent RNA2 bases found in DI-634. Regions in black (*B*) could be removed without destroying the ability of the DI to replicate. nt, Nucleotides.

Size as a Constraint on Efficient Encapsidation of RNA *in Vivo*. Six spontaneously arising DI RNAs derived from FHV RNA2 ranged in size from 620 to 680 bases (data not shown), suggesting a size constraint among viral RNAs for either replication or encapsidation. Further evidence that size did indeed influence packaging efficiency was obtained through studies on two DI clones constructed by restoring 155 (DI-789) or 267 (DI-901) RNA2 bases to DI-634 (Fig. 2*A*). These larger DI clones were created by *in vitro* recombination using cDNAs of FHV RNA2 and DI-634; transcripts from these and other cDNA constructs were generated *in vitro* from their linearized DNA templates using T3 RNA polymerase.

To compare the replication of DI-634 with its elongated versions, we used Lipofectin-mediated transfection (24) to introduce RNA transcripts from each clone into *Drosophila* cells together with FHV RNA1. As shown in Fig. 2*B*, DI-789 (lane 2) and DI-901 (lane 3) were replicated as efficiently as DI-634 (lane 1), indicating that insertion of sequences between DI segments did not impair replication. The fainter bands migrating between RNA1 and the DI RNAs correspond to double-stranded DI forms and (in the case of lane 3) to contaminating RNA2.

Because of the large deletions, none of the three DI RNAs encodes the coat protein needed for virion assembly. Thus, to examine their packaging activity, an experiment similar to that

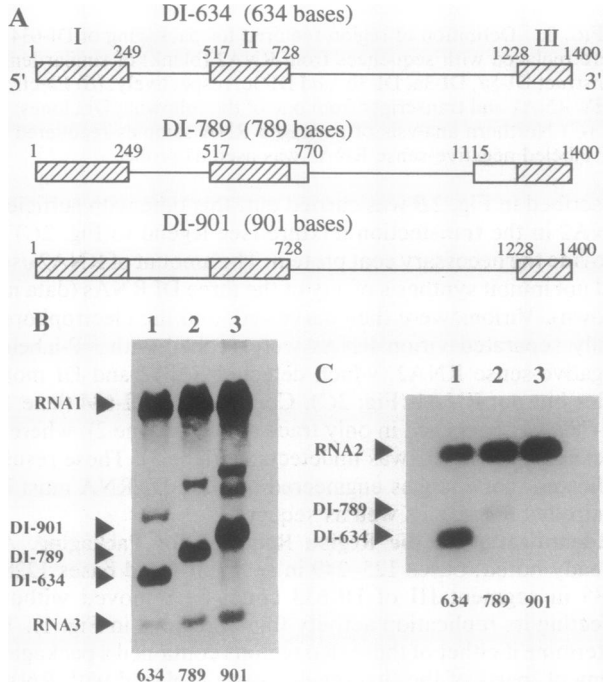

FIG. 2. Evidence that size is a constraint on DI-634 encapsidation. (*A*) Structures of two DI clones with additional RNA2 sequences (shown in blank) engineered between segments II and III (DI-789) or between I and II (DI-901). The base numbers represent their original positions in RNA2 sequence. (*B*) Electropherogram of [^{3}H]uridine-labeled RNA from *Drosophila* cells cotransfected with transcripts (DI-634, lane 1; DI-789, lane 2; or DI-901, lane 3) plus FHV RNA1. Forty nanograms of purified RNA1 and 20 ng of respective DI transcripts were used for transfection of 2×10^6 cells. About 20 hr after infection, newly synthesized RNAs were labeled by incorporation of [5,6-^{3}H]uridine (100 μCi/ml) in the presence of actinomycin D (12 μg/ml) for a 2-hr period. Total RNAs were then extracted and samples corresponding to 4×10^5 cells were separated on a 1.4% agarose gel. (*C*) Northern blot analysis of RNA molecules packaged in virus particles. Virions were harvested from 8×10^6 *Drosophila* cells cotransfected with 250 ng of FHV RNA1, 50 ng of RNA2, and 100 ng of respective DI transcripts. RNA2 was included to provide coat protein necessary for virion assembly. ^{32}P-labeled negative-sense RNA2 was used as probe.

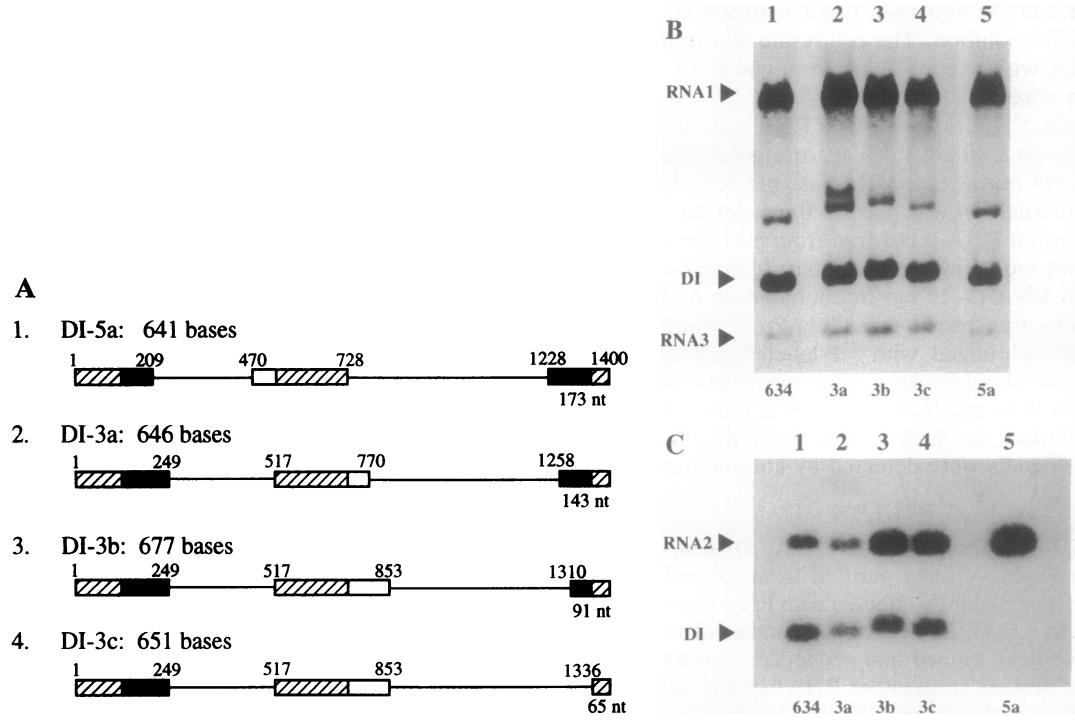

FIG. 3. Definition of region required for packaging of DI-634. (*A*) Structures of DI clones. Parts of replication-dispensible regions (black) were replaced with sequences from RNA2 (blank) of similar length. Bases 210–249, 1228–1257, 1228–1309, and 1228–1335 were replaced to construct DI-5a, DI-3a, DI-3b, and DI-3c, respectively. (*B*) Electropherogram of [^{3}H]uridine-labeled RNA samples from cells cotransfected with FHV RNA1 and transcripts from one of the following DI clones: DI-634 (lane 1), DI-3a (lane 2), DI-3b (lane 3), DI-3c (lane 4), and DI-5a (lane 5). (*C*) Northern analysis of packaged RNA samples recovered from cells cotransfected with RNA1, RNA2, and respective DI transcripts. ^{32}P-labeled negative-sense RNA2 was used as probe.

described in Fig. 2*B* was carried out, this time with sufficient RNA2 in the transfection mixture (see legend to Fig. 2*C*) to provide the necessary coat protein. The amount of RNA2 used did not inhibit synthesis of any of the three DI RNAs (data not shown). Virions were then harvested, and the electrophoretically separated virion RNAs were probed with ^{32}P-labeled negative-sense RNA2, which detected RNA2 and DI molecules but not RNA1 (Fig. 2*C*). Compared to DI-634 (lane 1), DI-789 was packaged in only trace amounts (lane 2), whereas packaging of DI-901 was undetectable (lane 3). These results indicated that changes engineered into the DI RNA must be controlled for size as well as sequence.

Identification of the Region Required for Packaging. As already noted, bases 125–249 in segment I and bases 1228–1335 in segment III of DI-634 could be removed without affecting its replication activity (black regions in Fig. 1). To determine if either of these two regions contained a packaging element, parts of the two regions were replaced with RNA2 sequences of similar size (Fig. 3*A*). DI-5a was made by replacing bases 210–249 with bases 470–516 from RNA2 (blank region); clones DI-3a, DI-3b, and DI-3c were similarly constructed with substitutions in the 3′ replication-dispensible region (see legend to Fig. 3).

As shown in Fig. 3*B*, transcripts from the above DI clones (lanes 2–5) were replicated as efficiently as those from DI-634 (lane 1) when cotransfected into *Drosophila* cells with FHV RNA1. Fig. 3*C* shows analysis of RNA molecules isolated from purified virions. Similar to DI-634 (lane 1), transcripts from DI-3a, DI-3b, and DI-3c were efficiently packaged (lanes 2, 3, and 4, respectively), demonstrating that the 3′ replication-dispensible region of DI-634 was not required for encapsidation. The slightly lower intensity of bands in lanes 1 and 2 reflects experimental variation in transfection efficiency and was not consistently observed in other experiments. Thus the proportion of DI to RNA2 is a more significant indicator of encapsidation than the intensity of bands.

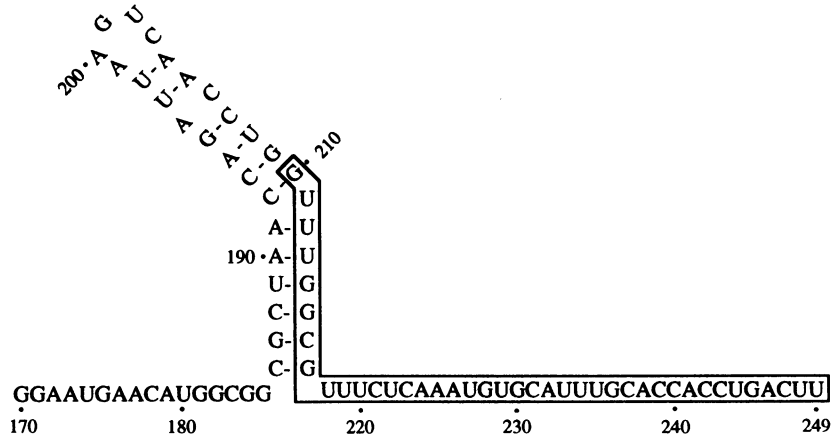

FIG. 4. Computer predicted stem–loop structure in the 5′ region of DI-634. The putative stem–loop, formed approximately between bases 186 and 217, consists of a 5-base loop and a 13-bp bulged stem with a free energy of −11.7 kcal/mol (1 kcal = 4.18 kJ) (Genetics Computer Group) (25). Bases 210–249 (boxed), essential for packaging, contain part of the stem–loop.

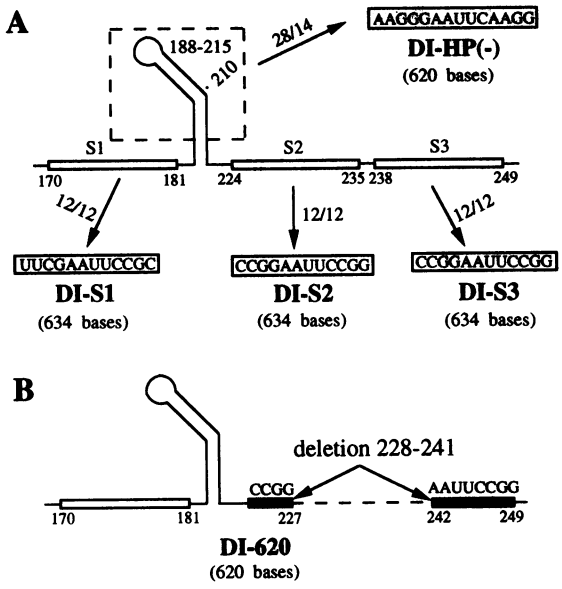

FIG. 5. Oligonucleotide-directed mutagenesis of sequences within or immediately adjacent to the predicted stem–loop structure. (*A*) Three 12-base sequences, S1 (GGAAUGAACAUG), S2 (AAAUGUGAAUUU), and S3 (ACCACCUGACUU), were separately replaced with a nonspecific synthetic linker of identical length to produce DI-S1, DI-S2, and DI-S3 clones. The region forming the predicted stem–loop (bases 186–217) remains intact. In DI-HP(−), however, most of the stem–loop (bases 188–215; 28 nt) was replaced with a 14-base linker designed not to form any secondary structure; DI-HP(−) is thus 14 bases shorter than DI-634. (*B*) DI-620: a 14-base deletion was made between bases 228 and 241 of DI-634. To make such a small deletion, two *Eco*RI sites were introduced into DI-634 clone at positions 228 and 241 followed by *Eco*RI digestion and religation of the resulting linear fragment. Regions in black represent the several base substitutions created during the process.

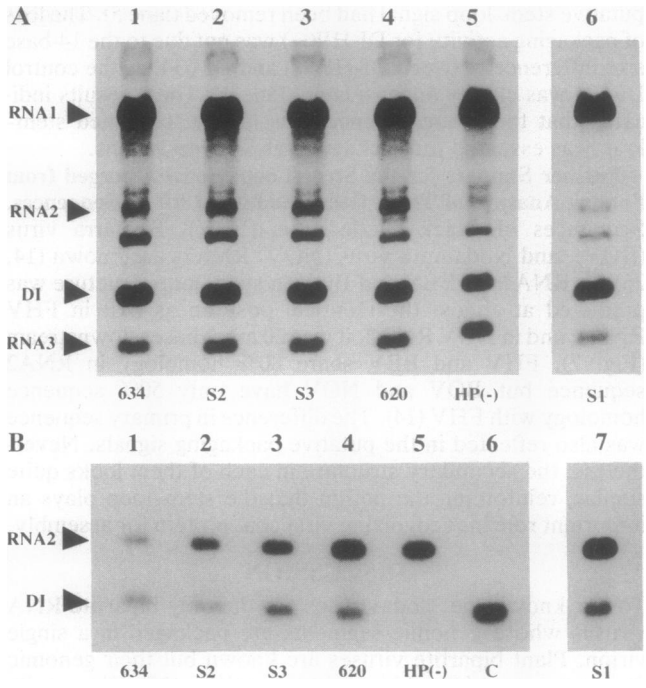

FIG. 6. Replication (*A*) and encapsidation (*B*) assays for transcripts from DI-S1, DI-S2, DI-S3, DI-620, and DI-HP(−). (*A*) Electropherogram of [^{3}H]uridine-labeled RNA samples isolated from cells cotransfected with RNA1, RNA2, and one of the following DI transcripts: DI-634 (lane 1), DI-S2 (lane 2), DI-S3 (lane 3), DI-620 (lane 4), DI-HP(−) (lane 5), and DI-S1 (lane 6). (*B*) Northern analysis of RNAs packaged into virus particles recovered from cells cotransfected with RNA1, RNA2, and the following DI transcripts: DI-634 (lane 1), DI-S2 (lane 2), DI-S3 (lane 3), DI-620 (lane 4), DI-HP(−) (lane 5), and DI-S1 (lane 7). In lane 6, DI-HP(−) molecules transcribed *in vitro* from cDNA were loaded directly onto the gel.

In sharp contrast, transcripts from DI-5a, with a 40-base deletion (bases 210–249) in the 5′ region, were not packaged (lane 5), indicating that bases 210–249 contained at least part of the essential element for encapsidation of DI-634.

Bases 210–249 Are Part of a Putative Stem–Loop Structure. Computer-assisted RNA folding analysis predicted that bases 210–249 are part of a stem–loop structure, formed between bases 186 and 217 with a 5-base loop and a 13-base-pair (bp) stem including a bulged "U" residue and a mismatched "A-C" base pair (Fig. 4).

To further examine the role of the predicted stem–loop in RNA packaging, we separately replaced four sequences in this region (Fig. 5*A*). Three 12-base regions (S1, S2, and S3) immediately adjacent to the stem–loop were substituted with synthetic oligonucleotide linkers of identical size to create DI-S1, DI-S2, and DI-S3. Part of the stem–loop, 28 bases (188–215), was replaced with a 14-base linker designed not to form any hairpin-like structure; thus the hairpinless DI clone, called DI-HP(−), is 14 bases shorter than DI-634. As a size control, a fifth clone (DI-620) was constructed with a 14-base deletion including parts of S2 and S3 regions (Fig. 5*B*). Transcripts from the modified DI clones, except those from DI-HP(−), retained the predicted stem–loop structure.

Transcripts from the above DI clones were all replicated efficiently in *Drosophila* cells with the help of FHV RNA1 (Fig. 6*A*); note the presence of RNA2 did not interfere with DI replication. As shown in Fig. 6*B*, all DIs were packaged into virions (lanes 2–4 and 7), except those from DI-HP(−), whose

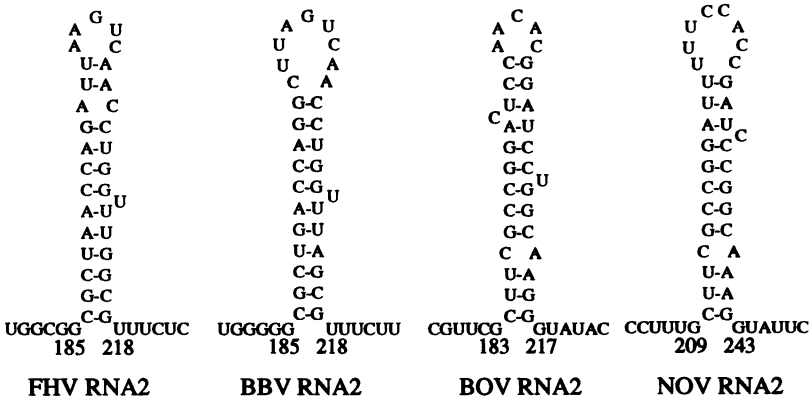

FIG. 7. Prediction of secondary structures in RNA2s of BBV, BOV, and NOV at similar locations as the packaging-essential element in FHV RNA2. BBV, BOV, and NOV RNA2s are 1399, 1305, and 1335 bases in length, respectively (14, 26).

putative stem–loop signal had been removed (lane 5). The loss of packaging activity for DI-HP(−) was not due to the 14-base size difference between DI-HP(−) and DI-634, as the control DI-620 was clearly encapsidated (lane 4). These results indicated that the sequence encompassing the predicted stem–loop was essential for packaging DI-634 into virions.

Further Support for the Stem–Loop Model Emerged from Folding Analysis of Three Other Nodaviral RNA2 Sequences. Sequences of Black beetle virus (BBV), Boolarra virus (BOV), and Nodamura virus (NOV) RNA2s are known (14, 26). In RNA2s of BBV and BOV, a stem–loop structure was predicted at almost the identical position as that in FHV RNA2; and in NOV RNA2, it was 20 more bases downstream (Fig. 7). FHV and BBV share 80% homology in RNA2 sequence but BOV and NOV have only 50% sequence homology with FHV (14). The difference in primary sequence was also reflected in the putative packaging signals. Nevertheless, the secondary structure in each of them looks quite similar, reinforcing the notion that the stem–loop plays an important role in recognizing viral coat protein for assembly.

DISCUSSION

To our knowledge, nodaviruses are the only bipartite RNA viruses whose genomic segments are packaged in a single virion. Plant bipartite viruses are known but their genomic parts are encapsidated in separate particles (27). The results presented in this report show that a specific region of FHV RNA2 (bases 186–217) with a predicted stem–loop structure was essential for *in vivo* encapsidation of a DI RNA (DI-634) derived from RNA2. Experiments are necessary to determine whether RNA1 also has a similar encapsidation signal. However, even if both RNAs are shown to contain separate encapsidation signals, this alone is not sufficient to explain how one of each RNA is selected for packaging. Some additional constraint(s), such as RNA complementarity, RNA–protein recognition elements, or need for a "headful" of RNA, must be involved in the packaging process.

A headful constraint for packaging, normally involving one RNA1 (3106 bases) plus one RNA2 (1400 bases), might, for example, be met by replacing RNA1 or RNA2 with equivalent masses of DI RNA. Our unpublished observations that six DIs related to RNA2 and one related to RNA1, which have been isolated so far, are all about half the length of the respective genomic RNA suggest definite size constraints on encapsidation of FHV RNA; the poor packaging of elongated forms of DI-634 is also consistent with the idea.

Specific regions of viral RNA genomes have been demonstrated to bind with coat protein in several positive-stranded RNA viruses and bacteriophages. In tobacco mosaic virus (TMV), a stem–loop structure consisting of 75 bases has long been known to be the precise origin of assembly (28, 29). Turner *et al.* (30) have shown that, in TMV, the putative secondary structure plays an important part in permitting selective protein–RNA recognition. Altering the RNA folding using site-directed mutagenesis reduced the rate of disk binding; so did shortening of the stem itself. Primary sequence changes, while maintaining the putative secondary structure, did not affect the specificity of coat protein–RNA interaction. It is also reported that regions with specific secondary structure in RNAs of alfalfa mosaic virus were used for binding with coat protein (31, 32). In *Escherichia coli* RNA bacteriophages R17 and Qβ, specific RNA regions with stem–loop structure plus several single-stranded bases have been documented to be essential for binding with coat protein (33–39). Coronavirus (40) and the L-A virus of yeast (41) also use stem–loops as packaging signals. Our results suggest that a similar strategy for recognition of viral genomic RNAs by coat protein exists in nodaviruses.

Identification of the packaging signal raises the possibility of using FHV as a vector for packaging messengers other than viral RNA. It remains to be seen, however, whether this region by itself is sufficient for encapsidation. An *in vitro* assembly assay may allow us to determine if foreign RNAs of appropriate size (up to 4500 bases) can be encapsidated by grafting the stem–loop into it.

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