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Packaging of Hepatitis Delta Virus RNA via the RNA-Binding Domain of Hepatitis Delta Antigens: Different Roles for the Small and Large Delta Antigens

HSEI-WEI WANG,[1] PEI-JER CHEN,[2,3] CHA-ZE LEE,[2] HUI-LIN WU,[3] AND DING-SHINN CHEN[3]*

Graduate Institutes of Microbiology[1] and Clinical Medicine,[2] College of Medicine, National Taiwan University, and Hepatitis Research Center, National Taiwan University Hospital,[3] Taipei, Taiwan

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Hepatitis delta virus (HDV) is composed of four specific components. The first component is envelope protein which contains hepatitis B surface antigens. The second and third components are nucleocapsid proteins, referred to as small and large hepatitis delta antigens (HDAgs). The final component is a single-stranded circular RNA molecule known as the viral genome. In order to study the mechanism of HDV RNA packaging, a four-plasmid cotransfection system in which each viral component was provided by a separate plasmid was employed. Virus-like particles released from Huh-7 cells receiving such a cotransfection were found to contain HDV RNA along with three proteins. Therefore, the four-plasmid cotransfection system could lead to successful HDV RNA packaging in vitro. The system was then used to show that the large HDAg alone was able to achieve a low level of HDV RNA packaging. Analysis of a variety of large HDAg mutants revealed that the RNA-binding domain was essential for viral RNA packaging. By increasing the incorporation of small HDAg into virus-like particles, we found a three- to fourfold enhancement of HDV RNA packaging. This effect was probably through a direct binding of HDV RNA, independent from that of large HDAg, with the small HDAg. The subsequent RNA-protein complex was packaged into particles. The results provided insight into the roles and functional domains of small and large HDAgs in HDV RNA packaging.

The virion of hepatitis D virus (HDV) is a 36-nm particle consisting of four specific components (1, 15). The envelope is composed of hepatitis B surface antigens (HBsAgs) provided by the helper hepatitis B virus (HBV) (1, 16). Inside the virion are two HDV-specific antigens, namely, the large and small hepatitis delta antigens (HDAgs) (1, 15). Also inside the virion is the 1.7-kb single-stranded circular RNA genome (15, 23). The assembly of these components into a mature virion is addressed in this study.

The in vitro production of HDV in tissue culture has been accomplished by the cotransfection of HDV cDNA and HBV DNA in Huh-7 cell lines (16, 24). It is therefore a useful system to study the components participating in HDV assembly. The HBsAgs are shown to be essential for HDV virion formation, (16, 19), with the smallest form of HBsAg being sufficient to accomplish virion assembly (22). However, the large form of HBsAg is required for infectivity of HDV virions (18). Regarding the function of these two HDV-specific antigens, the large HDAg is essential for HDV assembly (3) and can be packaged into HBsAg particles by itself (6, 16). In contrast, the small HDAg alone could not be packaged into HBsAg particles but depends on a protein-protein interaction with the large HDAg (6, 11). The primary role of each viral protein in HDV virion assembly is known, although details of their function merit further investigation. However, packaging of the central component of HDV, the viral genome (HDV RNA), is poorly understood.

Factors involved in packaging of the HDV genome probably can be divided into two groups. One is the *trans*-acting components, such as viral small and large HDAgs or cellular factors. The other is the *cis*-acting elements, such as RNA conformation or the nucleic acid packaging signals. In order to study these components, we first had to establish an in vitro HDV RNA packaging system. From previous work, we and others showed that by cotransfection of three plasmids (each expressing HBsAgs and large or small HDAg, respectively) into Huh-7 cells, the three proteins could assemble into virus-like particles which contain no viral RNA (6, 16). Branching out from this system, we considered whether further cotransfection with an additional plasmid expressing the full-length HDV RNA could lead to packaging of viral RNA into particles. If such a experiment worked, it could be used to study the role of each specific component of the HDV virion in RNA packaging.

Plasmids supplying the HBsAg and large and small HDAgs are already available (3, 10, 22). However, a plasmid which provides HDV RNA has not been perfected. Because wild-type HDV RNA itself can replicate after transfection into cells and subsequently generate both the large and small HDAgs (5, 8), it is not possible to address separately the roles of each individual HDAg in viral RNA packaging. To solve these problems, we followed a previous strategy and used a mutant HDV in which the HDAg open reading frame (ORF) was disrupted by a two-base deletion, thus destroying its self-replication capacity (10, 21). The mutant HDV cDNA was placed under the control of the potent cytomegalovirus (CMV) early promoter in order to maximize the level of RNA expression (2).

In this report, we present evidence that such a cotransfection system is successful in HDV RNA packaging in vitro. It was then used to dissect the role and protein functional domain of HDAgs involved in RNA packaging. The results advanced our understanding of the mechanism of HDV RNA packaging.

* Corresponding author. Mailing address: Hepatitis Research Center, National Taiwan University Hospital, 7 Chung-Shan South Rd., Taipei 100, Taiwan. Fax: 886-2-3317624.

MATERIALS AND METHODS

Plasmids. $pSVLD_3$ contains a tandem trimer of the full-length, wild-type HDV cDNA under the control of the simian virus 40 late promoter. It was used in transfection experiments to initiate viral genome replication and both large and small HDAg production (10). pSVDAg-L contains a *Bam*HI-*Pst*I subgenomic fragment of HDV cDNA under the control of the simian virus 40 promoter and expresses the large HDAg (3). pSVDAg-S is a plasmid containing a subgenomic HDV cDNA fragment and expresses the small HDAg only (10). Finally, pS_1X contains an HBV subgenomic fragment (*Apa*I-*Bgl*II) and was used to express three forms of HBsAg (22).

Construction of a plasmid expressing mutant HDV RNA. The construct was prepared as shown in Fig. 1A. In brief, an HDV cDNA monomer (pXX^-; 20) was cleaved at the unique *Sac*II site in the HDAg ORF (Fig. 1A) and then made blunt ended by treatment with Klenow fragment. After self-ligation and transformation into *Escherichia coli*, a plasmid with a deletion of two nucleotides at amino acid 10 of the HDAg coding region was obtained. The mutation was confirmed by sequencing analysis. A tandem dimer of such a mutant was cloned under the control of the human CMV immediate-early promoter in two orientations. One plasmid which expressed genomic HDV RNA (pCDm2G) was used in this study (Fig. 1A).

Construction of plasmids expressing mutant HDAgs defective in the oligomerization, RNA-binding, or carboxyl-terminal domain. Plasmids expressing mutant HDAgs with defective oligomerization capacity were kindly provided by Michael Lai (12, 25). Because of the substitution of two leucine residues in the coiled-coil structure (Fig. 1B, CCS) by glycine and valine (pSGVL) or by glycine and glycine (pSGGL), the oligomerization capacity of small HDAg is reduced about 50% in the former and almost completely in the latter (25). Two more plasmids, pLGVL and pLGGL, expressing mutant large HDAg with the above-mentioned defects, were also obtained (Fig. 1B) (25). A plasmid expressing mutant small HDAg defective in RNA binding was obtained from a previous study (12) in which two critical arginine residues in the first arginine-rich motif (ARM) of the RNA-binding domain of small HDAg were changed to glycine and glutamine (plasmid pdA1). The mutant small HDAg could not bind HDV RNA and could not support HDV RNA replication (12). In order to prepare a plasmid expressing mutant large HDAg with a similar defect, the *Sac*II-*Sma*I fragment (covering the RNA-binding domain) of this mutant plasmid was used to replace the corresponding part in the wild-type large HDAg to make plasmid pDA1 (Fig. 1B).

To construct a plasmid expressing mutant small HDAg truncated in the carboxyl terminus, pSVDAg-S (expressing wild-type small HDAg) was cleaved with *Sma*I at a position corresponding to amino acid 163 and then cleaved with *Bam*HI in the vector backbone. Afterwards the ends were repaired by filling in with Klenow fragment, and then they were self-ligated. The final plasmid, pddC, expressed a fusion protein containing the first 163 amino acids of small HDAg and 19 irrelevant amino acids derived from the vector sequences.

DNA transfection of Huh-7 cell lines. Transfection of Huh-7 cells was performed by the calcium phosphate precipitation method (14, 24). In general, 4×10^6 cells were seeded onto a 100-mm-diameter petri dish and were inoculated with a total of 40 μg of DNA. The amount of each plasmid used was 10 μg (unless specified in experiments), and salmon sperm DNA was used to keep the total amount of DNA per transfection constant. Culture medium was collected on days 3, 6, and 9 posttransfection to characterize the particles produced. The efficiency of transfection was monitored by determining the amount of HBsAg released into the medium by use of a commercial enzyme-linked immunoassay (IMX; Abbott). Quantitative comparisons of RNA packaging among particles produced in different cotransfection experiments varied by less than 10% in their transfection efficiency.

Purification and characterization of viral particles. Ten milliliters of culture medium was collected and then clarified by spinning at 12,000 rpm in a J2-21 centrifuge (Beckman) for 30 min. The supernatant was layered over a 20% sucrose cushion and then was spun down in an SW28 rotor at 25,000 rpm for 17 h (model L-8M; Beckman). The pellet was dispersed by repeated pipetting and dissolving in 150 to 200 μl of 10 mM Tris–1 mM EDTA (TE buffer) (adjusted to transfection efficiency). For isopycnic centrifugation, the dissolved pellet was put on top of a preformed discontinuous CsCl gradient (from 1.19 to 1.51 g/cm^3) in an SW41 rotor. After centrifugation at 35,000 rpm for 24 h (L8-M; Beckman), 0.5-ml fractions were collected from the bottom until the tube was empty. The refractive index of each fraction was determined by a refractometer and then was converted into density. To collect viral particles, 0.5 ml from each fraction was diluted with 6 volumes of phosphate-buffered saline and then centrifuged in a TLA100.3 rotor (TL-100; Beckman) at 60,000 rpm for 2 h. A dissolved pellet from each fraction was used for viral RNA and viral antigen detection to locate the fraction(s) containing HDV-like particles.

Northern (RNA) and Western immunoblotting procedures. Total RNA from transfected Huh-7 cells or from viral pellets was isolated by the single-step method (7). Northern blotting to detect HDV RNA and preparation of riboprobe were performed as previously described (3). Unless specified, the exposure time for autoradiography was 20 h (overnight). For protein detection, 30 μl of dissolved pellet was used to examine the viral antigen in the particles by Western blotting procedures (3, 6). The primary antibody was a high-titer human polyclonal antiserum against HDAgs, as used in a previous study (3). A horseradish peroxidase-conjugated sheep anti-human immunoglobulin antibody was used as the secondary antibody (Amersham). Reacted protein was detected by a chemiluminescence method (the ECL system from Amersham). The exposure time was 2 to 5 min. The signals of HDV RNA and HDAgs were traced by a densitometer for comparison (Bio-Rad model 620 video densitometer).

RESULTS

Production of the monomeric form of mutant HDV RNA in cells transfected with pCDm2G. In order to investigate the role of each viral component in the packaging of HDV RNA, we employed a cotransfection system in which each component of the HDV virion was supplied by an individual plasmid. Three plasmids were used previously to provide the HBsAgs and large and small HDAg, respectively (3, 10, 22). We then constructed a plasmid (pCDm2G) that transcribed the genomic strand of a tandem dimer of mutant HDV cDNA (two bases deleted in the HDAg gene) (Fig. 1A).

Plasmid pCDm2G was transfected into Huh-7 cells to test whether a full-length mutant HDV RNA could be produced. Total cellular RNA was extracted from Huh-7 cells at 3, 6, and 9 days posttransfection for Northern blot analysis. Signals of 1.7-kb monomeric HDV RNA were clearly seen (Fig. 2A, lanes 1 to 3). The results suggested that the primary transcript from pCDm2G (the 3.4-kb species in Fig. 2A, lanes 1 to 3) could undergo self-processing to generate a full-length, monomeric

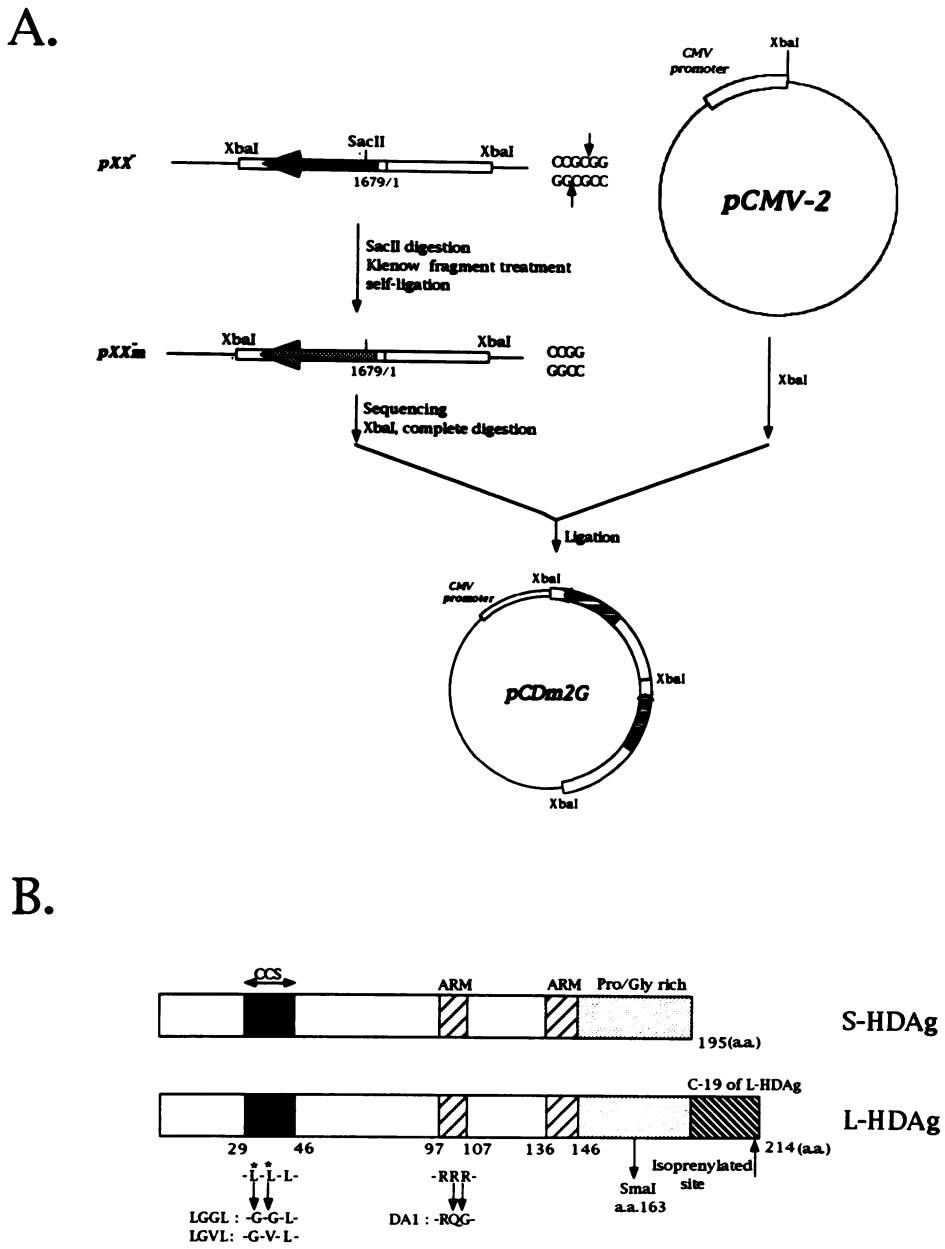

FIG. 1. (A) Diagram showing the construction of plasmids expressing the replication-defective HDV RNA. The thick open box in plasmid pXX⁻ represents the HDV sequence, the solid arrow represents the ORF of HDAg. A thin arrow on the sequence indicates the cleavage site of the *Sac*II enzyme. After a series of manipulations (as indicated in the text), two bases were deleted from the HDAg ORF and mutated sequences are shown to the right. The shaded arrow represents the mutated HDAg ORF. The mutated HDV cDNA was then cloned into the *Xba*I site of pCMV-2 in a tandem dimer. Under the control of the CMV early promoter, pCDm2G can transcribe genomic RNA. (B) Diagram showing the functional domains of small and large HDAgs (S- or L-HDAg, respectively) and important mutation sites. CCS, region of coiled-coil structure at the amino terminus; ARM, arginine-rich motif important for RNA binding. The isoprenylation site of large HDAg is indicated. The two leucine residues in the CCS are mutated to glycine and valine in mutant pLGVL but to glycine and glycine in mutant pLGGL. Two arginine residues in the first ARM of large HDAg are changed to G and Q to make the RNA-binding mutant pDA1. The small HDAg ORF was disrupted by *Sma*I digestion (at amino acid 163) to prepare a mutant with a truncated C terminus (pddC).

HDV RNA. The mutant HDV RNA alone, however, could not self-replicate because its amount declined gradually (Fig. 2A, lane 1 versus lane 3). To further confirm this, the same blot was stripped and then rehybridized with a probe detecting antigenomic RNA. In Huh-7 cells transfected with plasmid pCDm2G only, no antigenomic RNA was found (Fig. 2B, lanes 1 to 3). However, in cotransfection experiments with small HDAg-expressing plasmids, the mutant RNA could initiate replication (Fig. 2A, lanes 4 to 6) and produce antigenomic RNA (Fig. 2B, lanes 4 to 6).

A four-plasmid cotransfection system leading to successful HDV RNA packaging and revealing a significant role of large HDAg in RNA packaging. After showing that plasmid pCDm2G could produce monomeric HDV RNA, we then

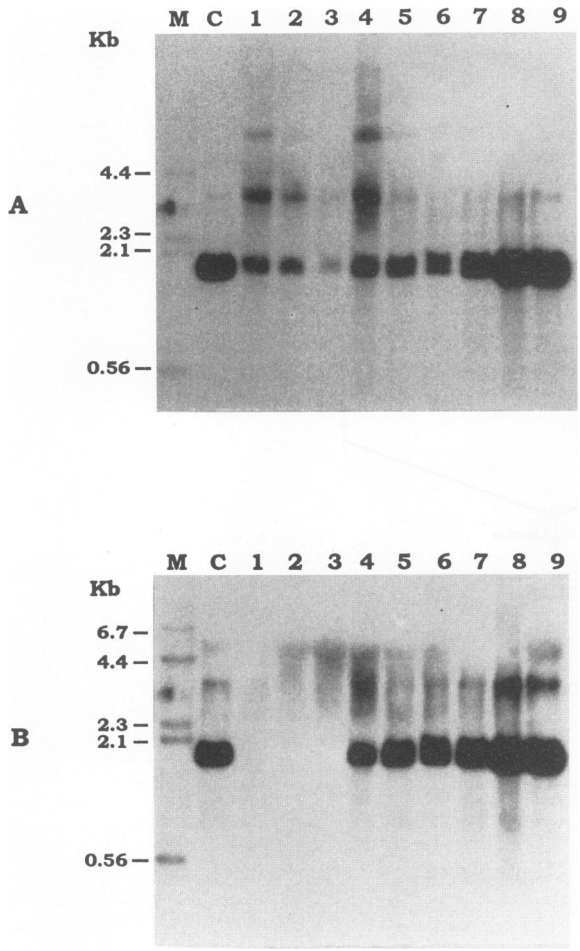

FIG. 2. Northern blot analysis of HDV RNA in Huh-7 cells transfected with plasmid expressing mutant HDV RNA. Huh-7 cells were transfected with pCDm2G alone (expressing mutant HDV RNA) (lanes 1 to 3), with pCDm2G plus pSVDAg-S (expressing small HDAg) (lanes 4 to 6), or with $pSVLD_3$ (expressing wild-type HDV RNA) (lanes 7 to 9). Total RNA was isolated from these cells at days 3, 6, and 9 posttransfection, and 20 μg was used for Northern blot analysis, either with a riboprobe detecting the genomic (A) or antigenomic (B) strand of HDV RNA. Lanes M, lambda *Hin*dIII fragments as markers; lanes C, 10 μg of total RNA extracted from Huh-7 cells cotransfected with $pSVLD_3$ plus pS_1X (expressing HBsAgs) as a positive control to show the position of the 1.7-kb HDV genome.

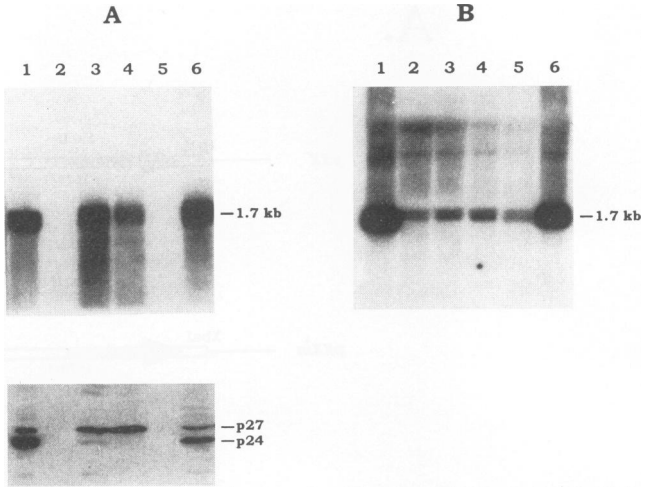

FIG. 3. Northern and Western blot analyses of HDV RNA and protein in virus-like particles and cells following cotransfection. (A) Detection of genomic HDV RNA in virus-like particles from the culture media of cotransfected Huh-7 cells. Culture media were collected from Huh-7 cells on day 6 posttransfection with pCDm2G (lane 2), with pCDm2G + pS_1X + pSVDAg-S (1 μg) + pSVDAg-L (10 μg) (lane 3), with pCDm2G + pS_1X + pSVDAg-L (lane 4), with pCDm2G + pS_1X + pSVDAg-S (lane 5), or with $pSVLD_3$ + pS_1X (lane 6). The amount of each plasmid used was 10 μg, unless specified. Virus-like particles were collected by centrifugation, and RNA was extracted from one-half of the pellet for Northern blot detection of packaged HDV RNA. Lane 1, 5 μg of total RNA extracted from Huh-7 cells transfected with $pSVLD_3$ as a size marker for monomeric HDV RNA. One-third of the same particles was used for immunoblot detection of HDAgs, and the results are shown at the bottom of panel A. Positions of the large (p27) and small (p24) HDAgs are indicated at the right. (B) Results of Northern blot analysis for genomic HDV RNA in cotransfected Huh-7 cells. Total cellular RNA was extracted from cotransfected Huh-7 cells, and 20 μg was used for Northern blot study. Samples in this panel are organized identically to those of panel A.

studied whether such a mutant RNA could be packaged. Four plasmids (expressing HBsAgs, large HDAg, small HDAg, or mutant HDV RNA) were cotransfected into Huh-7 cells. Four days after cotransfection, culture media were collected and virus-like particles were purified for HDV RNA and HDAg detection. Northern hybridization demonstrated HDV RNA in the particles (Fig. 3A, lane 3) which also contain both the large and small HDAgs (Fig. 3A, bottom gel, lane 3). Therefore, the results indicated that this mutant HDV RNA could be packaged into virus-like particles and suggested this experiment to be a practical in vitro HDV RNA packaging system.

Next, we investigated the role of individual HDAg in HDV RNA packaging. The large HDAg has been shown to be essential for HDV assembly (3) and itself could be packaged into HBsAg particles (6, 16). It was thus interesting to see whether the large HDAg alone could help HDV RNA packaging. To test this possibility, a similar cotransfection experiment was done, excluding the small HDAg-expressing plasmid. Culture media were collected from transfected Huh-7 cells, and virus-like particles were purified for the study of HDV components. As anticipated, large HDAg was packaged into the particles (Fig. 3A, bottom gel, lane 4). Interestingly, even without small HDAg, HDV RNA was still found in the purified particles (Fig. 3A, lane 4). This result suggested that large HDAg alone was sufficient in assisting HDV RNA packaging.

As negative controls, in the absence of HBsAg-expressing plasmid (Fig. 3A, lane 2) or large HDAg-expressing plasmid (lane 5), no HDV RNA or protein was packaged, although the level of HDV RNA inside the transfected cells was equal (Fig. 3B, lanes 2 and 5).

Isopycnic centrifugation analysis of virus-like particles for HDV RNA and HDAgs. In order to further demonstrate that HDV RNA was actually packaged into virus-like particles containing HDAgs, we banded the virus-like particles by isopycnic centrifugation in a cesium chloride gradient. The fractions were collected and analyzed for both HDV RNA and HDAgs. For particles containing both large and small HDAgs, fractions that contained HDAgs (Fig. 4A, lower gel, fractions 12 to 15) were coincidental with those that contained HDV RNA (Fig. 4A, upper gel, fractions 12 to 15). The peak fractions (13 and 14) had a density of 1.24 g/cm^3. These studies

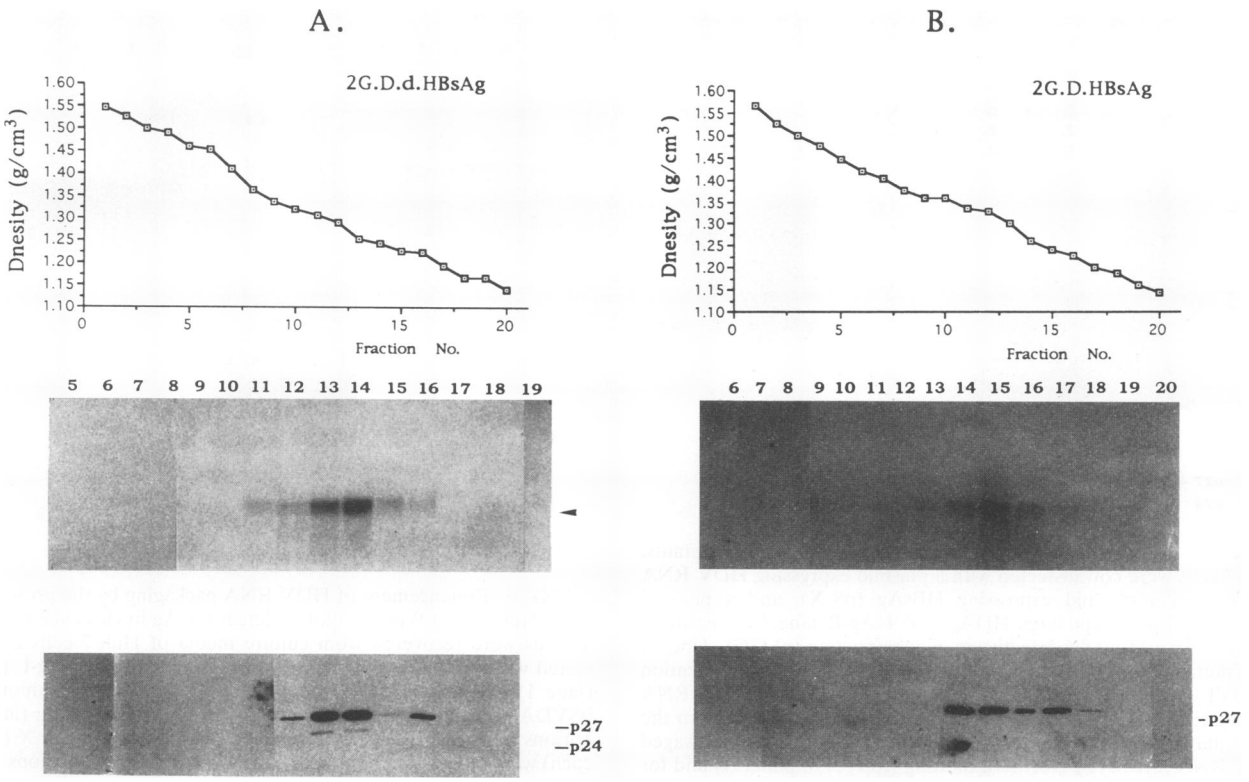

FIG. 4. Cesium chloride density gradient analysis of HDV-like particles for viral RNA and protein content. (A) Pellets from culture media of Huh-7 cells on day 3 posttransfection with pCDm2G (10 μg) + pS_1X (10 μg) + pSVDAg-L (5 μg) + pSVDAg-S (5 μg) were subjected to isopycnic centrifugation in a discontinuous CsCl gradient. The density profile is at the top. Each fraction was collected and subjected to Northern blot for detection of HDV (upper gel, autoradiography after exposure for 20 h) and Western blot for detection of large and small HDAgs (p27 and p24, lower gel). The size of monomeric HDV genome is indicated by an arrowhead. (B) Similar study conducted for isopycnic fractions of virus-like particles from Huh-7 cells cotransfected with pCDm2G + pS_1X + pSVDAg-L (10 μg each). The autoradiograph of the Northern blot was exposed for 5 days. Positions of HDV RNA and large HDAg (p27) are marked.

confirmed that HDV RNA was indeed packaged into particles in this cotransfection experiment.

A similar study was conducted on the particles containing large HDAg only. Pellets recovered from culture media of Huh-7 cells cotransfected with pCDm2G, pS_1X, and pSVDAg-L were subjected to isopycnic centrifugation. Immunoblotting analysis found that particles in fractions 14 to 18 contained large HDAg (Fig. 4B, lower gel). Northern blot analysis could detect HDV RNA in the same fractions (Fig. 4B, upper gel), with the peak fraction (no. 15) having a density of 1.24 g/cm^3. Such results indicated that HDV RNA could be packaged into HBsAg particles containing only large HDAg.

However, the efficiency of HDV RNA packaging in particles containing the large HDAg only was less than that in particles containing both large and small HDAgs. In the peak isopycnic fractions, the amount of particles containing only large HDAg appeared to be equivalent to that of particles containing both large and small HDAgs, as suggested by a similar level of immunoblotting signals (compare the p27 signal of fractions 14 and 15 of Fig. 4B with that of fractions 13 and 14 of Fig. 4A). Comparison by densitometer quantitation revealed a less than 20% difference. In contrast, the detection of HDV RNA in the corresponding fractions of the former particles required a sixfold longer exposure time (5 days) than did those of the latter particles (20 h). Even after prolonged exposure, their hybridization signals were still found to be 30 to 50% of those of the latter. The discrepancy might reflect a functional difference between large and small HDAg in HDV RNA packaging.

RNA-binding domain of large HDAg essential for HDV RNA packaging. After identifying a significant role of large HDAg in assisting viral RNA packaging, we then investigated its functional domain. On the basis of previous results, large HDAg probably can be divided into four domains with distinct functions. The amino-terminal coiled-coil structure (Fig. 1B, CCS) is an oligomerization domain mediating the protein-protein interaction (6, 11, 25). The middle region contains two arginine-rich motifs (Fig. 1B, ARM) and is critical for binding with HDV RNA. The importance of an RNA-binding domain in viral replication has been shown (4, 12, 13). The carboxyl-terminus is a region rich in proline and glycine, with unknown function. Finally, the C-terminal extreme of 19 amino acids is posttranslationally modified by isoprenylation and is found to be essential in virion assembly (9, 11). Large HDAg mutants which are defective in the C-terminal region performed poorly in self-packaging (11) and very likely even worse in assisting RNA packaging. We therefore only focused on the significance of the coiled-coil structure and the RNA-binding domain in RNA packaging.

Two coiled-coil domain mutants, pLGVL and pLGGL, were used. When two leucine residues in the coiled-coil structure are replaced (by glycine and valine or by glycine and glycine), the oligomerization activities of pLGVL and pLGGL are expected to diminish partially or almost completely (25). One

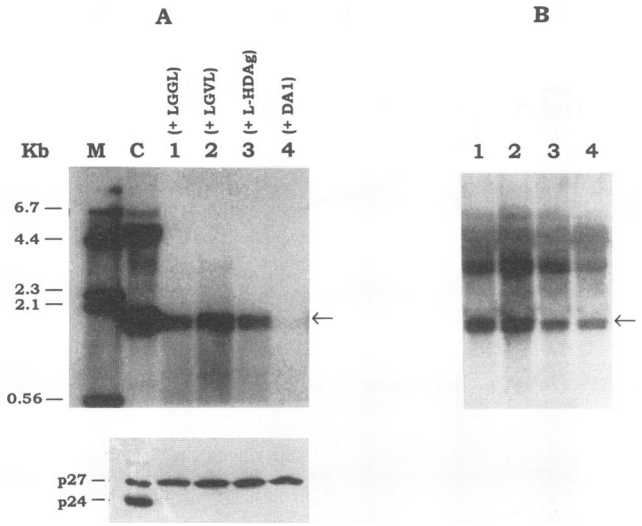

FIG. 5. Effect on HDV RNA packaging by large HDAg mutants. Huh-7 cells were cotransfected with a plasmid expressing HDV RNA (pCDm2G), a plasmid expressing HBsAg (pS_1X), and a plasmid expressing (i) wild-type large HDAg (pSVDAg-L, lane 3), (ii) mutant large HDAg profoundly defective in oligomerization (pLGGL, lane 1), (iii) mutant large HDAg partially defective in oligomerization (pLGVL, lane 2), or (iv) mutant large HDAg defective in RNA binding (pDA1, lane 4). Virus-like particles were recovered from the four kinds of cotransfected cells and used for detection of packaged HDV RNA (arrow) by Northern blotting (top gel of panel A) and for detection of HDAgs by immunoblotting (bottom gel of panel A). The wild-type (lane 3) and three mutant large HDAgs (lanes 1, 2, and 4) were found at similar levels. In contrast, little HDV RNA was packaged by mutant large HDAg defective in the RNA-binding domain (lane 4). Lanes M and C were the same as described in the legend to Fig. 2. (B) Northern blotting showing the amount of HDV RNA in the cotransfected Huh-7 cells. The samples were arranged in a same way as described for panel A.

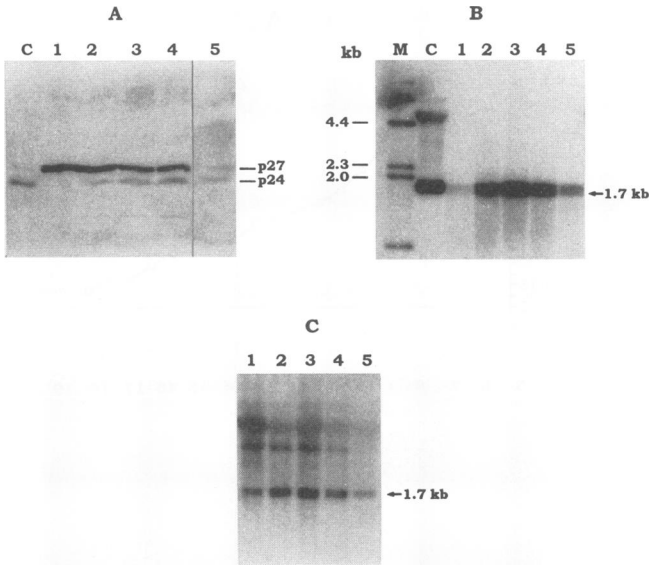

FIG. 6. Enhancement of HDV RNA packaging by the presence of small HDAg. (A) Western blot to detect HDAg in virus-like particles. Virions were recovered from culture media of Huh-7 cells cotransfected with pCDm2G (10 μg) + pS_1X (10 μg) + pSVDAg-L (5 μg) (lane 1) or the above three plasmids plus an increasing amount of pSVDAg-S from 5 μg (lane 2), to 10 μg (lane 3), to 15 μg (lane 4). Virions from Huh-7 cells cotransfected with $pSVLD_3$ + pS_1X (10 μg each) were included as a positive control (lane 5). The positions of the large (p27) and small (p24) HDAgs are at the right. Lane C, two species of HDAg in the HDV virion used for markers. (B and C) Northern blot of the HDV genome in the pellets from the experiment shown in panel A (B) or in cotransfected cells (C). The organization of these panels was identical to that described in the legend to panel A. Arrows, monomer RNA. Lanes M and C, HDAg markers.

RNA-binding domain mutant, pDA1, was also used. When two arginines in the first ARM are replaced, RNA-binding activity is essentially abolished (12).

The capacity of these three mutants to assist HDV RNA packaging was tested by cotransfecting each plasmid with two other plasmids producing HDV RNA (pCDm2G) and expressing HBsAg (pS_1X) in Huh-7 cells. Virus-like particles were obtained from culture media by centrifugation. The three mutant large HDAgs could be packaged into particles, as shown by immunoblotting studies (Fig. 5A, bottom gel, lanes 1, 2, and 4). After quantitation of the intensity of the protein signal, all three mutants were found to be packaged with an efficiency equal to that of wild-type large HDAg (Fig. 5A, bottom gel, lane 3). Such particles were then tested for their HDV RNA content by Northern blot analysis. HDV RNA was detected at similar levels in particles containing wild-type large HDAg (Fig. 5A, lane 3) and containing oligomerization mutants (pLGGL or pLGVL). However, in particles containing RNA-binding domain mutant pDA1 there was little HDV RNA (Fig. 5A, lane 4). As a control for comparison, the HDV RNA in transfected cells was investigated by Northern blotting analysis (Fig. 5B, arrow). After normalization of packaged RNA to the level of cellular RNA, the efficiency of mutant large HDAg (pDA1) in RNA packaging was found to be less than 5% of the other three. Clearly, the large HDAg defective in RNA binding was ineffective in assisting HDV RNA packaging.

Enhancing role of small HDAg in HDV RNA packaging. Both large and small HDAgs are present in HDV virions, suggesting that both are important in RNA packaging. Comparison of HDV RNA levels in particles containing both HDAgs with those of particles containing only large HDAg revealed a significant difference. In the latter group of particles, the amount of packaged HDV RNA was much less (compare middle gel of Fig. 4B with that of Fig. 4A). This observation was consistent with an ineffective packaging of HDV RNA in the absence of small HDAg.

To better understand the role of small HDAg in RNA packaging, we did a titration experiment. The amounts of HDV RNA-, HBsAg-, and large HDAg-expressing plasmids were fixed, whereas that of small HDAg-expressing plasmid was serially increased. However, the amount of small HDAg-expressing plasmid was always kept below that of the large HDAg-expressing plasmids. This precaution was taken to prevent RNA replication, thereby ensuring that the HDV RNA produced was dependent exclusively on the levels of transfected plasmids. (As noted from previous studies, under such a ratio of large to small HDAg little HDV RNA replication could take place [5].)

Pellets from culture media of Huh-7 cells thus transfected were prepared and used for immunoblotting to detect the HDAgs. For the four transfection experiments, the amounts of large HDAg-containing particles were equal (Fig. 6A, p27 in lanes 1 to 4). The amounts of small HDAg in particles, however, gradually increased in the last three samples (Fig 6A, p24 in lanes 2 to 4) and paralleled the increase in the amount

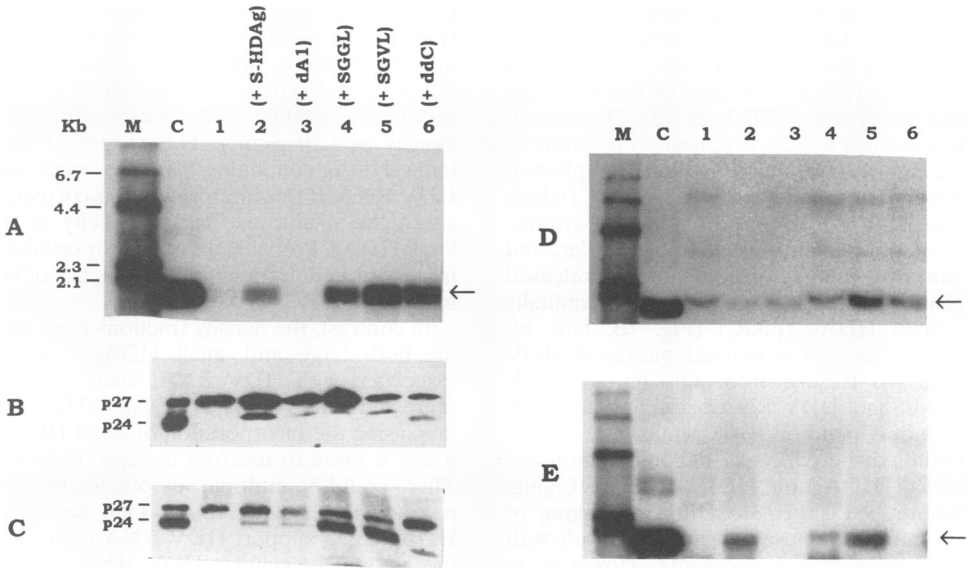

FIG. 7. Effect on HDV RNA packaging by wild-type and mutant small HDAg mutants. Huh-7 cells were cotransfected with pCDm2G, pS_1X, and pDA1 (expressing mutant large HDAg defective in RNA packaging) and no other plasmid (lane 1) or with a plasmid expressing wild-type small HDAg (lane 2), mutant small HDAg defective in the RNA-binding domain (pdA1, lane 3), mutant small HDAg defective in the oligomerization domain (pSGGL, lane 4), mutant small HDAg partially defective in oligomerization (pSGVL, lane 5), or mutant small HDAg with a C-terminal truncation (pddC, lane 6). Culture media and cells were collected at day 6 posttransfection for analysis of HDV. Virus-like particles were purified from culture media, and RNAs were extracted for Northern blot detection of packaged HDV RNA (A). Results for immunoblot detection of HDAgs in particles (B) and those for detection of expressed HDAgs in transfected cells (C) are shown. Results of riboprobe detection of genomic HDV RNA (D) or antigenomic RNA (E) in transfected cells are shown. Lanes M, lambda *Hin*dIII fragments as size markers; lanes C, HDV RNA as markers.

of transfected small HDAg-expressing plasmid. Therefore, the incorporation of small HDAg into particles increased as anticipated.

We then determined the RNA content in these four groups by hybridization with probes specific to HDV genomic RNA. The amount of packaged HDV RNA was the least in particles containing only large HDAg (Fig. 6B, lane 1). When small HDAg was added to the particles, the amount of packaged HDV RNA increased significantly (Fig. 6B, lanes 2 to 4). Densitometer tracings of the HDV RNA hybridization signals indicated a 6 to 10-fold increase in RNA packaging. To compare the packaging efficiency more correctly, the relative ratio of the RNA hybridization signal of the particles should be further normalized to that of cellular HDV RNA (Fig. 6C, lanes 1 to 4). After this conversion, the efficiency of HDV RNA packaging was found to increase to 4.2-fold when the ratio of small to large HDAg was at 0.12, 4.6-fold with a ratio at 0.36, and 4.4-fold at a ratio of 0.5. Thus, even a low level of incorporation of small HDAg was effective in increasing the efficiency of HDV RNA packaging, suggesting an important role for small HDAg in RNA packaging.

HDV RNA packaging through direct binding with small HDAg. In the above experiments, the particles containing both HDAgs exhibited better HDV RNA packaging than those containing only large HDAg. This raised the possibility that small HDAg has an additional mechanism for RNA packaging. To test this possibility, we used the mutant large HDAg, pDA1, which had almost no RNA packaging activity, as shown in previous cotransfection experiments (Fig. 5A, lane 4). The advantage of using this mutant is that it allowed us to exclude RNA packaging by large HDAg, thus simply addressing whether small HDAg possessed independent RNA packaging capacity.

Huh-7 cells were cotransfected with three plasmids expressing HDV RNA (pCDm2G), HBsAg (pS_1X), and mutant large HDAg defective in RNA packaging (pDA1) and with a plasmid expressing either wild-type small HDAg or a variety of mutant small HDAgs. Particles were purified from culture media of the cotransfected cells and then subjected to immunoblotting and Northern blot analysis.

The particles containing mutant large HDAg defective in RNA binding (pDA1) (Fig. 7B, p27 in lane 1) were unable to package HDV RNA (Fig. 7A, lane 1). However, if the particles also contained wild-type small HDAg (Fig. 7B, p24 in lane 2), packaging of viral RNA could be recovered (Fig. 7A, lane 2). The results clearly demonstrate that small HDAg has an RNA packaging capacity independent of that of large HDAg. This RNA packaging could take place first through direct HDV RNA binding with small HDAg and then by a mutant large HDAg interaction through its intact oligomerization domain (3, 11). To further test this hypothesis, a mutant small HDAg defective in RNA binding (pdA1), instead of the wild-type small HDAg, was used in cotransfection. The mutant small HDAg was packaged into particles (Fig. 7B, lane 3), but it was unable to recover RNA packaging (Fig. 7A, lane 3). These data indicate that one mechanism for better HDV RNA packaging in particles containing both large and small HDAgs could be an additional, independent RNA packaging ability by small HDAg.

However, an alternative explanation is that it is secondary to an increase of cellular HDV RNA due to viral replication in the presence of small HDAg. In cotransfection experiments with wild-type small HDAg, although we kept the ratio of large to small HDAg at a level unfavorable to HDV RNA replication (5), it was difficult to eliminate this possibility. As microheterogeneity in mixtures of large to small HDAg-expressing

plasmids could occur, some cells might experience a change in the ratio of large to small HDAg which would allow some RNA replication. In fact, by the presence of antigenomic RNA in cotransfected cells we detected a low level of RNA replication in cells receiving pDA1 and pSVDAg-S (Fig. 7E, lane 2). In order to exclude the effect of RNA replication resulting in better RNA packaging, we further constructed a plasmid expressing a C-terminally truncated small HDAg (pddC) which was unable to support HDV RNA replication. In cells cotransfected with this mutant, only genomic HDV RNA was detected (Fig. 7D and E, lanes 6). Characterization of the released particles revealed that there was packaging of the C-terminally truncated mutant small HDAg (pddC) (Fig. 7B, lane 6). Interestingly, such particles still contained packaged HDV RNA (Fig. 7A, lane 6). Results from this mutant study suggested that the additional HDV RNA packaging ability by the small HDAg did not depend on RNA replication.

Finally, we also studied the effects of oligomerization domain mutants of small HDAg on HDV RNA packaging. Before the experiments, we anticipated little packaging of HDV RNA by these mutants because they interact poorly with the large HDAg and thus hinder packaging. However, in studying the protein components of the released particles, we did find packaging of a small amount of mutant small HDAg, either pSGGL or pSGVL (Fig. 7B, lanes 4 and 5). It was noteworthy that in cell lysates the amount of mutant small HDAg pSGGL or pSGVL was more abundant than that of large HDAg (Fig. 7C, lanes 4 and 5), just the opposite of the levels in the particles. The results were consistent with a sharp decline, but not a complete loss, of oligomerization activity of such mutants. Nevertheless, such mutant small HDAgs, once packaged into particles, retained their ability to package HDV RNA, as shown in Fig. 7A, lanes 4 and 5.

DISCUSSION

Previous studies have shown that HBsAgs and large and small HDAgs, coexpressed in the Huh-7 cells, can be assembled into empty HDV-like particles that contain no viral RNA (6, 16). Extending from such results, we added to the cotransfection experiment another plasmid expressing a mutant HDV RNA (2-base deletion in the HDAg ORF) to study HDV RNA packaging. It was shown in this four-plasmid cotransfection system that HDV RNA could be packaged (Fig. 3A, lane 3) into virus-like particles.

With this in vitro packaging system, the four specific components of the HDV virion could be provided separately, thus allowing us to examine the role of each individual component in RNA packaging. When small HDAg was left out of the cotransfection, HDV RNA could still be packaged (Fig. 3A, lane 4). This finding was further confirmed by isopycnic centrifugation study of these particles, showing that fractions containing the large HDAg and HDV RNA were coincident (upper gel of Fig. 4B). Therefore, large HDAg alone was able to bring HDV RNA into virus-like particles. Furthermore, we demonstrated that the RNA-binding domain was critical for its role in RNA packaging. Once mutated, the mutant large HDAg lost its RNA packaging ability, despite normal packaging of mutant protein itself (Fig. 5A, lane 4). In contrast, the oligomerization domain seemed unrelated to HDV RNA packaging ability. Our results indicate a common RNA-binding domain involved in two important steps of the HDV life cycle, namely, RNA replication and packaging.

Although the large HDAg alone could mediate packaging of HDV RNA, the efficiency was low. From isopycnic centrifugation studies, fractions corresponding to large HDAg-containing particles carry much less HDV RNA than fractions corresponding to particles containing both HDAgs (compare the upper gels of Fig. 4A and B). Actually, we noted a separate, minor peak fraction which appeared to contain large HDAg only but little HDV RNA (fraction 17 in Fig. 4A and B, with a density of 1.20 g/cm^3). This suggested that a proportion of large HDAg-containing particles were probably devoid of HDV RNA. If this interpretation is correct, it raises a question about the significance and specificity of RNA packaging by large HDAg. Probably other viral or cellular factors were more important in determining the efficiency of HDV RNA packaging.

In contrast, the density fractions from the particles containing both large and small HDAgs appeared always to be associated with HDV RNA. Such observations suggested a more active role of small HDAg in HDV RNA packaging. By increasing the incorporation of small HDAg into particles, we found a three to fourfold increase in HDV RNA packaging. There could be multiple reasons for the more effective RNA packaging by small HDAg. One possibility was that small HDAg could support HDV RNA replication, thus increasing the amount of cellular HDV RNA available for packaging. However, this was not supported for two reasons. (i) The comparison of packaged RNA was normalized to that of cellular RNA, and (ii) the mutant small HDAg unable to support HDV replication (pddC) was still able to enhance HDV RNA packaging (data not shown).

A second possibility was that small HDAg possessed a better RNA-binding activity than did large HDAg. However, in previous Northwestern studies, the in vitro RNA-binding activities between large and small HDAgs were shown to be equivalent (12, 13) and therefore could not explain the difference seen in packaging. Another possibility was a difference in accessibility of HDV RNA inside the cells. Actually, in transfected or infected cells, most of the HDV RNA was found to be associated with small HDAg not with large HDAg (17). This compartmentalization effect could be a factor in deciding the relative packaging efficiency of both HDAgs.

Finally, we provided experimental data supporting one simple mechanism for this enhancement effect. This was shown to be a direct binding of HDV RNA to small HDAg, independent from that of the large HDAg. In an experimental condition that prevented RNA packaging through large HDAg, we found that small HDAg could still package HDV RNA. The HDV RNA probably bound with small HDAg first, through the RNA-binding domain of the protein. Then the ribonucleoprotein complex was introduced into the particle via a protein-protein interaction with large HDAg. This possibility was supported by the analysis of another RNA-binding domain mutant of small HDAg. This mechanism probably occurred naturally in HDV RNA packaging. A recent study of HDV RNA in infected cells found little free HDV RNA, with most existing as a ribonucleoprotein complex (17). Viral protein in such a ribonucleoprotein complex was found almost exclusively in the small HDAg (17). If the precursor for virion RNA (packaged RNA) was this ribonucleoprotein (inside the cells, there was little HDV RNA existing in other form), packaging probably proceeded through a protein interaction with the large HDAg, as demonstrated in the above experiment (Fig. 7).

During the study, we found that the two oligomerization domain mutants still retain some protein-protein interaction capacity, although sharply decreased (especially mutant pSGGL). They could be packaged by a weak interaction with the large HDAg and they could even support a low level of RNA replication. Probably such mutations did not abolish the

oligomerization activity completely or there might also be a minor protein-protein interaction domain other than the coiled-coil structure. As has been noted previously, there is a putative helix-loop-helix structure in the central RNA-binding domain (4) which could be a candidate for a minor oligomerization domain.

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