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Functional Domains of Delta Antigens and Viral RNA Required for RNA Packaging of Hepatitis Delta Virus

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The functions of delta antigens (HDAgs) in the morphogenesis of hepatitis delta virus (HDV) have been studied previously. The C terminus of large HDAg has been shown to complex with the small surface antigen (HBsAg) of helper hepatitis B virus, whereas the assembly of small HDAg requires interaction with the N terminus of large HDAg (M.-F. Chang, C.-J. Chen, and S. C. Chang, J. Virol. 68:646–653, 1994). To further examine the molecular mechanisms by which HDAgs are involved in the assembly of HDV RNA, we have cotransfected Huh-7 cells with plasmids representing a longer than unit-length HDV and the small HBsAg cDNAs. We found that HDAg mRNA could be generated from an endogenous promoter within the HDV cDNA that was translated into large HDAg. Large HDAg is capable of complexing with monomeric HDV genomic RNA to form ribonucleoprotein particles (RNPs) and is capable of forming enveloped HDV-like particles in the presence of small HBsAg without undergoing HDV replication. In addition, the middle region from amino acid residues 89 to 145 of large HDAg is required for assembly of the RNPs but is dispensable for assembly of the enveloped particles. RNA assembly is also demonstrated with small HDAg when it is cotransfected with a packaging-defective large HDAg mutant and small HBsAg. Leu-115 within the putative helix-loop-helix structure of the small HDAg is important for the replication of HDV but is not essential for RNA assembly, suggesting that conformational requirements of small HDAg for replication and assembly of viral RNA may be different. Further studies indicate that a 312-nucleotide linear HDV RNA from one end of the HDV rod structure is sufficient to form RNP complexes competent for assembly of virus-like particles with large HDAg and small HBsAg.

Hepatitis delta virus (HDV) possesses a unique genomic structure that does not resemble that of any known animal viruses (26, 39). The mature virion consists of a closed circular RNA genome approximately 1.7 kb in length and virus-specific delta antigens (small and large HDAgs) that are encapsidated by the surface antigens (HBsAgs) of the helper hepatitis B virus (1, 2, 4, 16, 30, 40). The large (pre-S1) but not the middle (pre-S2) form of HBsAg is essential for HDV infectivity (35, 36). However, the middle form of HBsAg is capable of interacting with small HDAg and may be involved in viral particle formation (12). The small form of HBsAg (major S) contains information sufficient for producing virus-like particles in the presence of large HDAg (3, 31, 37). Furthermore, recent results indicated that sequences within the C-terminal 19-amino-acid domain flanking the Cxxx isoprenylation motif of large HDAg are essential for the formation of virus-like particles with small HBsAg (6, 14, 15, 21). In addition, two functional motifs within the N-terminal 65 amino acid residues of large HDAg are important for the assembly of small HDAg and *trans* repression of viral replication (6, 9, 19, 38, 42). Nevertheless, the involvement of HDAgs in the RNA packaging of HDV is not fully understood.

HDV RNA undergoes self-cleavage during replication to produce a unit-length, unbranched rod-like structure (7, 16). Although a host DNA-dependent RNA polymerase II-like enzyme may be involved in HDV replication, the enzyme has not yet been identified, and the molecular mechanisms of small HDAg in the transactivation of HDV replication are not clear (13, 17, 25). One possible function of small HDAg is to localize HDV RNA to the nucleus through an interaction between the RNA and small HDAg (5, 43). This hypothesis is supported by the fact that HDAgs can complex with HDV RNA to form ribonucleoprotein particles (RNPs) both in viral particles and in transfected cells (23, 32). In addition, RNA-binding activity has been demonstrated for small HDAg; Leu-115 within the putative helix-loop-helix structure and two arginine-rich motifs in the middle domain of small HDAg were identified as important for binding activity as well as for the replication of HDV (4, 7, 22, 23).

In this report, we characterize the functional domains of HDAgs and viral RNA that are involved in the RNA packaging of HDV. Results indicate that the middle domain of large HDAg is essential for the packaging of HDV RNA. In addition, small HDAg is capable of copackaging with HDV genomic RNA in the presence of small HBsAg and a packaging-defective large HDAg mutant. A site-directed mutagenesis at Leu-115 results in a failure of small HDAg to support the replication of HDV but has no significant effect on packaging of the viral genomic RNA. Moreover, a linear HDV RNA containing a putative rod-like structure from nucleotides (nt) 654 to 965 is sufficient for viral RNA assembly.

MATERIALS AND METHODS

Cell line and DNA-RNA transfection. Huh-7 (human hepatoma) cells were cultured at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum plus 100 U of penicillin and 100 μg of streptomycin per ml. DNA and RNA transfections were performed with cationic liposomes as described by the manufacturer (Bethesda Research Laboratories).

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Plasmid construction and in vitro RNA synthesis. (i) Plasmids pDPS22, pCMV2025, and pCMV2025d*Sac*II. To facilitate the construction of plasmid pDPS22, recombinant plasmid pTZ-d-PS was first generated by inserting the 317-bp *Pst*I-*Sal*I DNA fragment of plasmid pT7-d-BP (4) into a modified pTZ19U vector (U.S. Biochemicals) in which the polylinker sequences from the *Pst*I site to the *Sal*I site had been deleted. A *Pst*I-*Pst*I monomeric HDV genomic cDNA fragment (26) was then subcloned into the *Pst*I site of plasmid pTZ-d-PS to generate plasmid pDPS22, which now contains a 2,025-bp, longer than unit-length HDV cDNA. For construction of plasmid pCMV2025, a *Hin*dIII-*Xba*I fragment from plasmid pDPS22 was subcloned into a modified pRc/CMV vector (Invitrogen) in which the polylinker sequences from the *Hin*dIII site to the *Xba*I site had been deleted. The resultant plasmid, pCMV2025, contains the 2,025-bp, longer than unit-length HDV cDNA under the control of the cytomegalovirus promoter. For construction of pCMV2025d*Sac*II, the *Sac*II-*Sac*II monomeric HDV cDNA fragment from plasmid pCMV2025 was treated with T4 DNA polymerase and inserted back into a modified pCMV2025 from which the *Sac*II-*Sac*II DNA fragment had been deleted and in which the remaining DNA had been blunted at the *Sac*II site by T4 DNA polymerase. The resultant plasmid, pCMV2025d*Sac*II, contains a 2,021-bp, longer than unit-length HDV cDNA with a modified reading frame which encodes an HDAg from amino acid residues 1 to 10 followed by 51 unrelated amino acid residues. Specific mutations in plasmid pCMV2025d*Sac*II and the following constructs used in this study were confirmed by DNA sequencing, using the dideoxy-chain termination method (34).

(ii) Plasmid pECE-C-ES. Plasmid pECE-C-ES encodes the small form of HBsAg as previously described (6).

(iii) Plasmids pECE-d-BE, pECEL-d35/88, pECEL-d89/163, and pECEL-d164/195. Plasmids pECE-d-BE, pECEL-d35/88, pECEL-d89/163, and pECEL-d164/195 encode wild-type large HDAg and its mutants with deletions from amino acid residues 35 to 88, 89 to 163, and 164 to 195, respectively, as previously described (4, 6).

(iv) Plasmid pECEL-d89/145. For construction of plasmid pECEL-d89/145, plasmid pECE-d-BE containing the *Bam*HI-*Eco*RI fragment of HDV cDNA was digested with *Stu*I plus *Nae*I before self-ligation. The resultant plasmid, pECEL-d89/145, encodes a large HDAg with a deletion from amino acid residues 89 to 145.

(v) Plasmids pECE-d-SM and pECE-d-SM(M). Plasmids pECE-d-SM and pECE-d-SM(M) encode wild-type small HDAg and its mutated form with Leu-115 changed to valine, respectively, as described previously (7).

(vi) Plasmid pBA4 and BA RNA. For construction of plasmid pBA4, the 582-bp *Bst*BI-*Ava*I DNA fragment of plasmid pD2 (7) was inserted into a modified pGEM4Z (Promega) that had been treated with *Acc*I plus *Ava*I. Plasmid pBA4 contains HDV cDNA from nt 529 to 1110. For preparation of BA RNA, plasmid pBA4 was treated with *Asp* 718, and in vitro RNA synthesis was performed with T7 RNA polymerase as previously described (7).

(vii) Plasmid pST4 and PP RNA. For construction of plasmid pST4, the 438-bp *Pst*I DNA fragment of plasmid pD2 (7) was inserted into a modified pGEM4Z (Promega) that had been treated with *Pst*I. Plasmid pST4 contains HDV cDNA from nt 651 to 1088. By using T7 RNA polymerase, PP RNA was transcribed in vitro from plasmid pST4 following digestion with *Bam*HI.

(viii) Plasmid pCMV-PS and PS RNA. Recombinant plasmid pDPS-PS was first obtained for construction of plasmid pCMV-PS. To construct plasmid pDPS-PS, plasmid pDPS22 was digested with *Sal*I to remove a unit-length HDV cDNA. Plasmid pDPS-PS was then treated with *Xba*I plus *Hin*dIII to generate a 312-bp DNA fragment that was further introduced into a modified pCMV2025 from which the *Xba*I-*Hin*dIII fragment had been deleted. The resultant plasmid, pCMV-PS, contains HDV cDNA from nt 654 to 965. Using T7 RNA polymerase, PS RNA was transcribed in vitro from plasmid pCMV-PS linearized with *Xba*I.

(ix) PO RNA. For preparation of PO RNA, a DNA template containing the T7 RNA polymerase promoter was generated by PCR (33). In brief, the reaction mixture consisting of PCR buffer (Promega), 0.2 mM each deoxynucleoside triphosphate, primers T7 (5′-GTAATACGACTCACTATA-3′) and 111 (912-TTCCTCTTCGGcaCGGCATGGCAT-889; lowercase letters indicate mutated nucleotides, and the nucleotides are numbered as described previously [26]), 1 ng of pCMV-PS, and *Taq* DNA polymerase (Promega) was subjected to 30 cycles of amplification. The 259-bp PCR product representing HDV sequences from nt 654 to 912 was used as a template for in vitro transcription with T7 RNA polymerase to generate PO RNA.

Isolation of HDV-like particles by a CsCl gradient. Virus-like particles were isolated as previously described (6, 37), with modifications. In brief, culture medium was harvested 4 to 6 days posttransfection and clarified by spinning at 9,000 rpm in an RA-4F rotor (Kubota) for 10 min. The supernatant was layered over a 20% sucrose cushion (20% sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 0.1% bovine serum albumin) and then centrifuged at 40,000 rpm in an SW41 rotor (Beckman) for 5 h to pellet the virus-like particles. The pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and subjected to equilibrium centrifugation in a discontinuous CsCl gradient (1.1, 1.2, 1.3, and 1.4 g/cm^3) at 35,000 rpm in an SW41 rotor for 24 h. Fractions of 0.5 ml were then collected from the bottom of the centrifuge tube with a peristatic pump (Gilson), and the density of each fraction was determined by a refractometer (ATAGO). Fractions 9 to 16 representing a density range from 1.30 to 1.16 g/cm^3 were analyzed for the presence of HDV RNA, large HDAg, and small HBsAg.

Isolation of RNPs from transfected cells. HDV-specific RNPs were isolated as described previously (32), with modifications. In brief, transfected cells were washed twice with cold phosphate-buffered saline and disrupted with cold lysis buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100). The nuclei were then pelleted by a short spin (Kubota 1200) and resuspended in 100 μl of cold lysis buffer without Triton X-100. Following sonication for 1 min, the nuclear lysate was further analyzed for the presence of HDV-specific RNPs by immunoprecipitation and by immunoblot and Northern (RNA) blot analyses.

Immunoprecipitation of RNPs. The HDAg-specific immunoglobulin G (4) was incubated with protein A–Sepharose CL-4B (Pharmacia) in NTE-NP40 buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]) at room temperature for 1 h with rocking. The protein A-HDAg antibody complexes were washed once with NTE-NP40 buffer and then incubated in NTE1 buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) with the nuclear lysates of transfected cells in the presence of 1 mM dithiothreitol and 80 U of RNasin at 4°C for 5 h with rocking. The reaction mixtures were then washed once with NTE-NP40 buffer and three times with NTE2 buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.1 mM EDTA). The immunoprecipitated products were then analyzed for the presence of HDV RNA and large HDAg by Northern blot and immunoblot analyses, respectively.

Immunoblot analysis. Immunoblotting was performed as previously described (6). In brief, whole-cell extracts, protein lysates of virus-like particles, and RNPs were prepared 4 to 6 days posttransfection, separated by SDS-polyacrylamide gel electrophoresis, and electrotransferred onto an Immobilon-P membrane (Millipore). The membrane was then incubated with antibodies specific to HDAg (23) or to HBsAg (Dako).

Northern blot analysis. Northern blot analysis was performed as previously described (6, 11). The probes used were the genomic and antigenomic strands of HDV RNA that were in vitro transcribed from *Sal*I dimeric HDV cDNA plasmid pD2 (7) with SP6 RNA polymerase and with T7 RNA polymerase, respectively, in the presence of [α-^{32}P]UTP.

RESULTS

Establishment of a system for studying the role of HDAgs in the packaging of HDV RNA. To study the assembly of HDV RNA, plasmid pDPS22, which represents a longer than unit-length of HDV genomic RNA with two potential self-cleavage sites (Fig. 1A), was first examined for its ability to undergo self-cleavage. Following in vitro transcription with linearized pDPS22, a full-length monomeric HDV RNA of 1.7 kb and two additional RNA molecules of 286 and 56 nt were detected in the presence of magnesium ions (Fig. 1B and C). The specific cleavage of HDV RNA into a unit-length molecule is probably an essential step prior to virion assembly. Plasmid pDPS22 contains an open reading frame encoding large but not small HDAg, thus providing a useful system with which to study the role of large HDAg in the assembly of HDV. However, HDV RNA cannot replicate in the absence of small HDAg. To overcome this problem, the exogenous promoter from cytomegalovirus was used to construct plasmid pCMV2025 (Fig. 2A), which is capable of producing a large amount of HDV RNA without viral replication. To study subdomains of the large and small HDAgs essential for RNA packaging, the open reading frame of large HDAg within pCMV2025 was destroyed to generate a plasmid designated pCMV2025d*Sac*II. Wild-type or mutant large and small HDAgs were then supplied by cotransfecting culture cells with the HDAg-expressing plasmids. The presence or absence of HDV genomic RNA in cell lysates and virus-like particles was then evaluated for the requirements of small and large HDAgs in the assembly of viral RNA.

Wild-type large HDAg is capable of complexing with HDV genomic RNA to form RNPs. The role of large HDAg in the packaging of HDV RNA was first examined by cotransfecting Huh-7 cells with plasmids pCMV2025 and pECE-C-ES, encoding small HBsAg, in the presence or absence of the wild-type large HDAg-expressing plasmid pECE-d-BE. Results demonstrate that HDV genomic RNA is present both in virus-

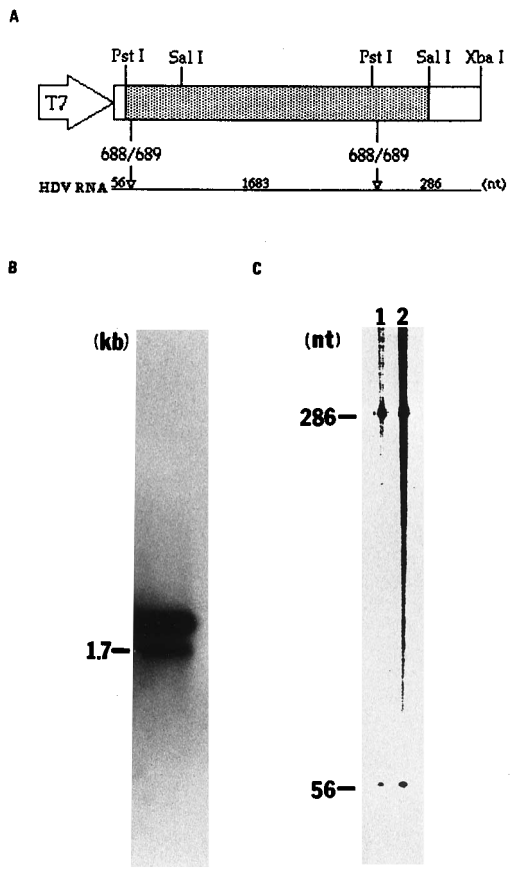

FIG. 1. Analysis of transcription products of plasmid pDPS22. (A) Diagram of the structure of plasmid pDPS22 and its corresponding transcript of a longer than unit-length HDV genomic RNA. The HDV cDNA is indicated by the stippled region. The promoter sequence of T7 RNA polymerase is indicated as T7, and the direction for transcription is indicated with an arrowhead. Two possible self-cleavage sites within the longer than unit-length HDV genomic RNA located between nt 688 and 689 are indicated. Self-cleavage generates a 1,683-nt monomeric HDV RNA and two additional products of 56 and 286 nt as indicated. (B) In vitro-transcribed HDV RNA products were analyzed by Northern blot analysis using the antigenomic strand of HDV RNA as previously described (6). The self-cleavage RNA product of approximately 1.7 kb is indicated. (C) The ^{32}P-labeled transcription products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Lanes 1 and 2 show duplicate reactions and the self-cleavage RNA products of 56 and 286 nt. Both in vitro transcription reactions were carried out with *Xba*I-linearized pDPS22, using T7 RNA polymerase in the absence (B) or presence (C) of [α-^{32}P]UTP.

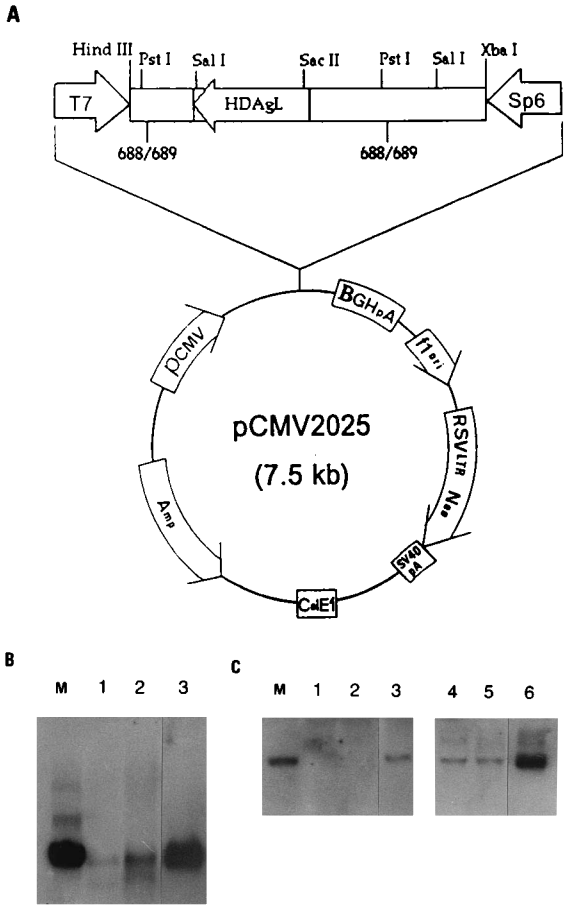

FIG. 2. Northern blot analysis of HDV RNA following cotransfection of Huh-7 cells with pCMV2025 and a plasmid encoding small HBsAg. (A) Schematic diagram of plasmid pCMV2025. Plasmid pCMV2025 contains sequences corresponding to a unit-length HDV cDNA (*Pst*I-*Pst*I fragment) plus an extra 317-bp *Pst*I-*Sal*I fragment. Two possible self-cleavage sites between nt 688 and 689 are indicated. The coding region of large HDAg is indicated by HDAgL. pCMV represents the promoter sequences of the immediately-early gene of human cytomegalovirus. BGHpA represents the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene. ori, origin of replication; RSVLTR, Rous sarcoma virus long terminal repeat; Neo, neomycin resistance gene; SV40 PA, simian virus 40 poly(A) site; Amp, ampicillin resistance gene. (B and C) Northern blot analysis of HDV RNA. Cotransfection studies were performed with plasmid pCMV2025 and plasmids representing small HBsAg (panel B, lane 1; panel C, lanes 1 and 4), small HBsAg plus large HDAg (panel B, lane 2; panel C, lanes 2 and 5), and small HBsAg plus small HDAg (panel B, lane 3; panel C, lanes 3 and 6). RNA samples were prepared from virus-like particles present in the culture medium (B) and from the transfected cells (C). The probes used were the antigenomic (panel B, lanes M and 1 to 3; panel C, lanes 4 to 6) and genomic (panel C, lanes 1 to 3 and M) strands of HDV RNA. Lanes M in panels B and C contain 1.7-kb in vitro transcripts of HDV genomic and antigenomic RNAs, respectively, that were hybridized to the HDV RNA probes as described in Materials and Methods and were used as markers.

like particles and in cell lysates even without cotransfecting culture cells with plasmid pECE-d-BE (Fig. 2B, lane 1; Fig. 2C, lane 4). This finding suggests the existence of an endogenous promoter within the HDV cDNA capable of driving transcription to produce mRNA for large HDAg in the antigenomic orientation. Large HDAg then serves to help package the viral RNA in the presence of small HBsAg. This hypothesis is supported by results of immunoblot analysis in which the wild-type large HDAg was detected in virus-like particles produced from Huh-7 cells transfected with plasmids pCMV2025 and pECE-C-ES (Fig. 3, lane 4). In addition, HDV antigenomic RNA was detected only in the presence of small HDAg (Fig. 2C, lane 3). Furthermore, the HDV RNA, large HDAg, and small HBsAg were shown to be present in the same fractions when virus-like particles isolated from the culture medium of transfected cells were further purified through a discontinuous CsCl gradient (Fig. 4). These results indicate that large HDAg is capable of complexing with the HDV genomic RNA to form RNPs and is sufficient to form HDV-like particles in the presence of small HBsAg in Huh-7 cells without undergoing viral replication.

A middle domain between amino acid residues 89 and 145 of large HDAg is essential for the formation of viral RNPs. To examine the region of large HDAg important for HDV RNA packaging, plasmids with various deletions within HDAg were constructed and coexpressed with small HBsAg and HDV RNA in Huh-7 cells. Neither the deletion near the N terminus encoded by pECEL-d35/88 (Fig. 5A, lane 3) nor the deletion

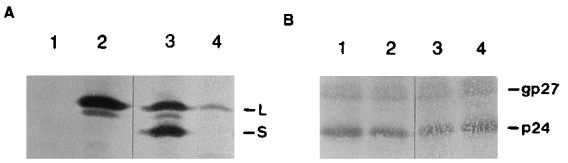

FIG. 3. Immunoblot analysis of HDAgs and small HBsAg following cotransfection of Huh-7 cells with a longer than unit-length HDV cDNA and plasmids representing small HBsAg and HDAgs. Cotransfection studies were carried out in Huh-7 cells with plasmid pECE-C-ES encoding small HBsAg and plasmids representing vector only (pECE; lanes 1), large HDAg (lanes 2), large HDAg plus small HDAg (lanes 3), and plasmid pCMV2025 containing a longer than unit-length HDV cDNA and the coding region of large HDAg (lanes 4). Protein lysates were prepared from virus-like particles secreted into culture media, and immunoblotting was carried out with antibodies specific to HDAgs (A) and to HBsAgs (B). Large and small HDAgs detected in the culture media are indicated by L and S, respectively. Two forms of small HBsAg, p24 and gp27, were identified as controls.

near the C terminus encoded by pECEL-d164/195 (Fig. 5A, lane 5) affected the ability of large HDAg to copackage with HDV genomic RNA. However, the deletion in the middle domain between amino acid residues 89 and 163 abolished the ability of large HDAg to copackage with HDV RNA (Fig. 5A, lane 4) even though the RNA was present in the transfected Huh-7 cells (Fig. 5B, lane 4). In addition, a large HDAg mutant with a deletion in the middle domain between amino acid residues 89 and 145 failed to form RNPs in transfected cells (Fig. 6, lanes LM), whereas both the wild-type large and small HDAgs formed RNPs with HDV RNA (Fig. 6, lanes L and S). Furthermore, both of the large HDAg mutants with deletions in the middle domain formed virus-like particles with small HBsAg (Fig. 7A, lanes 5 and 6). These results indicate that the middle domain between amino acid residues 89 and 145 of large HDAg, a region containing arginine-rich motifs (22), is important for complexing with HDV RNA to form RNPs but is dispensable for assembly of enveloped particles. This finding is consistent with an in vitro study demonstrating that the middle domain of HDAg contains RNA-binding activity (4, 23).

Small HDAg is capable of copackaging with HDV RNA in the presence of small HBsAg and a large HDAg mutant with deletions in the RNA-binding domain. Large HDAg mutants with deletions in the middle RNA-binding domain are capable of copackaging with the wild-type small HDAg and small HBsAg (Fig. 7A, lanes 2 and 3), although the mutant large HDAg failed to copackage the viral RNA (Fig. 5A, lane 4). Therefore, a cotransfection system with plasmid pCMV2025d*Sac*II and plasmids representing small HBsAg, copackaging-defective large HDAg, and small HDAg of wild-type or mutated form was chosen to study the characteristics of small HDAg in copackaging HDV RNA. Plasmid pCMV2025d*Sac*II represents a longer than unit-length HDV genomic RNA that lacks the open reading frame encoding either wild-type small or wild-type large HDAg. HDV genomic RNA was detected by Northern blot analysis in both the virus-like particles (Fig. 7B, lane 1) and transfected Huh-7 cells (Fig. 7B, lane 5) when the cotransfection was performed with wild-type small HDAg. In addition, the antigenomic RNA was also detected in transfected cells (Fig. 7B, lane 3). These results suggest that wild-type small HDAg can compensate for a mutation in the RNA-binding domain of large HDAg to copackage HDV RNA.

Leu-115 of the small HDAg is essential for viral replication but not for assembly of HDV RNA. The middle domains between amino acid residues 89 and 163 of large (Fig. 5) and small (data not shown) HDAgs play important roles in the assembly of HDV RNA. In addition, Leu-115 within the helix-loop-helix structure of the middle domain of small HDAg is essential both for RNA binding in vitro and for viral replication in Huh-7 cells (7) (Fig. 7B, lanes 3 and 4). However, whether Leu-115 of small HDAg is important for the assembly of HDV RNA is unknown. By cotransfecting Huh-7 cells with pCMV2025d*Sac*II and plasmids representing small HBsAg, the large HDAg mutant with a deletion in the RNA-binding domain, and a small HDAg with the Leu-115 mutation, we detected HDV RNA in both the virus-like particles (Fig. 7B, lane 2) and the cell lysate (Fig. 7B, lane 6). This finding indicates that Leu-115 of small HDAg is dispensable for copackaging of HDV RNA with the HDAgs.

A linear HDV RNA fragment from nt 654 to 965 is capable of copackaging with large HDAg and small HBsAg into virus-like particles. To examine the packaging signal of HDV RNA, linear viral RNAs of various lengths were in vitro transcribed

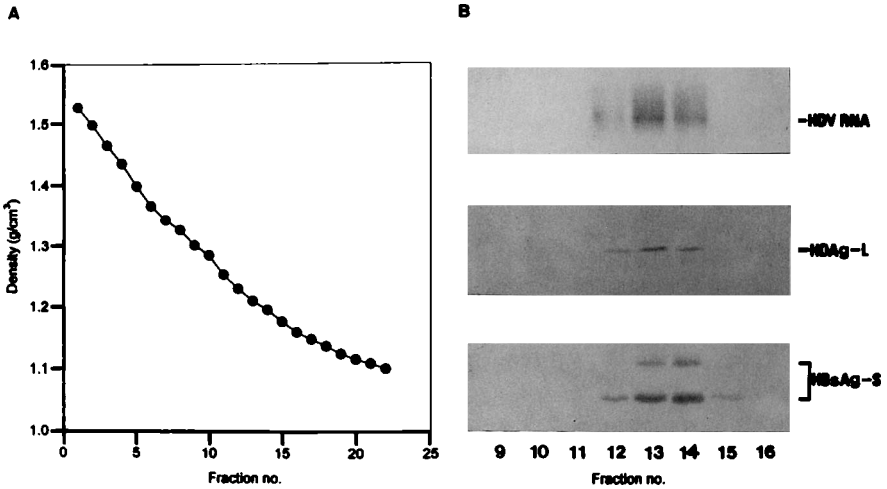

FIG. 4. Analysis of HDV-like particles. (A) CsCl gradient for isolating HDV-like particles. HDV-like particles were isolated from the culture medium of Huh-7 cells transfected with pCMV2025 and pECE-d-BE by a discontinuous CsCl gradient as described in Materials and Methods. (B) Analyses of HDV RNA, large HDAg, and small HBsAg. Fractions 9 to 16 of the CsCl gradient were analyzed for the presence of HDV RNA (top), large HDAg (middle), and small HBsAgs (p24 and gp27) (bottom). Fraction 13 contains the majority of HDV-like particles and has a density of around 1.21 g/cm^3.

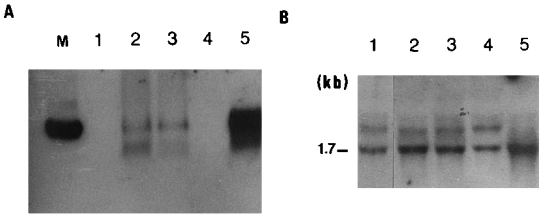

FIG. 5. Northern blot analysis of HDV RNA following cotransfection of Huh-7 cells with a longer than unit-length HDV cDNA and plasmids encoding small HBsAg and large HDAg mutants. Plasmids used in the cotransfection studies were pCMV2025d*Sac*II (lanes 1), pCMV2025d*Sac*II plus either the wild-type large HDAg (lanes 2), or large HDAg mutants that were deleted between amino acid residues 35 and 88 (lanes 3), 89 and 163 (lanes 4), or 164 and 195 (lanes 5). The HDV RNA of virus-like particles (A) and total cellular lysates (B) were isolated 6 days posttransfection. The probe used was the antigenomic strand of HDV RNA and was prepared as described in Materials and Methods. An in vitro transcript representing the 1.7-kb monomeric HDV genomic RNA hybridized to the antigenomic RNA probe was used as a marker (panel A, lane M; panel B as indicated).

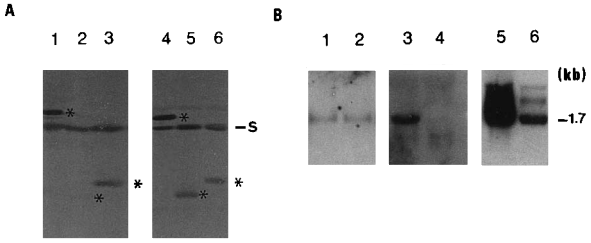

FIG. 7. Analysis of HDAgs and HDV RNA following cotransfection of Huh-7 cells with plasmids encoding small HBsAg, HDAg mutants, and a longer than unit-length HDV cDNA. (A) Immunoblot analysis of HDAgs. HDAgs expressed in transfected Huh-7 cells were wild-type small HDAg and large HDAgs of the wild-type form (lanes 1 and 4) and mutants that were deleted between amino acid residues 89 and 163 (lanes 2 and 5) and between amino acid residues 89 and 145 (lanes 3 and 6). Protein lysates were prepared from the virus-like particles secreted into culture media (lanes 1 to 3) or from transfected cells (lanes 4 to 6). Immunoblots were performed with antibodies specific to HDAgs. Wild-type small HDAg is indicated by S, and wild-type large HDAg and its mutants are indicated by asterisks. (B) Northern blot analysis of HDV RNA. Plasmids used in the cotransfection studies were pCMV2025d*Sac*II, pECEL-d89/163 (a large HDAg mutant with deletion between amino acid residues 89 and 163), and plasmids representing small HDAg of the wild-type form (lanes 1, 3, and 5) and a mutant with a single amino acid change at Leu-115 (lanes 2, 4, and 6). RNA samples were prepared 6 days posttransfection from virus-like particles (lanes 1 and 2) and transfected cells (lanes 3 to 6). The probes used were the antigenomic (lanes 1, 2, 5, and 6) and the genomic (lanes 3 and 4) strands of HDV RNA and were prepared as described in Materials and Methods. The 1.7-kb monomeric HDV RNA detected in both virus-like particles and cell lysates is indicated.

and cotransfected into Huh-7 cells with plasmids encoding large HDAg and small HBsAg. Results in Fig. 8C show that the HDV RNA fragments containing sequences from nt 529 to 1110 (lane 1), 651 to 1088 (lane 2), and 654 to 965 (lane 3) were copackaged efficiently into virus-like particles. The 259-nt HDV RNA from nt 654 to 912 was copackaged less efficiently (lane 4). Nevertheless, in the transfected Huh-7 cells, the 259-nt HDV RNA was present at higher amount than the other RNA fragments (Fig. 8B). These results indicate that a linear viral RNA with 312 nt from 654 to 965 containing a putative rod-like structure is sufficient for the packaging of HDV RNA.

DISCUSSION

We have recently shown that sequences within the C-terminal 19-amino-acid domain containing the isoprenylation motif are important for the assembly of large HDAg (6). In addition, the N-terminal 65-amino-acid domain is important for the assembly of small HDAg (6). In this study, we demonstrate that the middle domain between amino acid residues 89 and 145 of large HDAg is important for complexing with HDV genomic RNA to form RNPs in transfected cells (Fig. 6). Furthermore, wild-type small HDAg can help an RNA packaging-defective large HDAg in the assembly of HDV RNA (Fig. 7A). These results indicate that both small and large HDAgs can form RNP complexes with HDV RNA and may be cooperative in viral RNA assembly. One observation to support this hypothesis is that almost equal amount of both HDAgs are present in the HDV virion (8). Our previous studies also demonstrated that the RNA-binding domain is localized in the middle one-third of HDAgs (23) and that Leu-115 within the putative helix-loop-helix structure and arginine-rich motifs of small HDAg are important for the RNA binding (7, 22). Although a

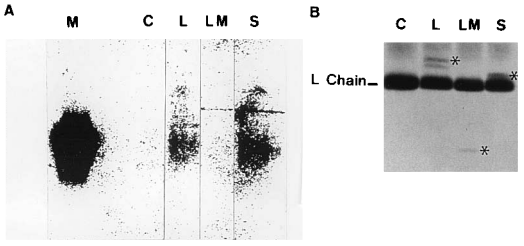

FIG. 6. Analysis of HDV RNP complexes from nuclei of transfected cells. (A) Northern blot analysis of HDV RNPs isolated from nuclei of transfected cells. Huh-7 cells were transfected with plasmid pCMV2025 plus either the pECE vector only (C), pECE-d-BE (L), pECEL-d89/145 (LM), or pECE-d-SM (S). Lane M contains the 1.7-kb in vitro transcripts of HDV genomic RNA and was used as a marker. After hybridization with the antigenomic RNA probe, the signals for HDV genomic RNA were analyzed with a PhosphoImager (Molecular Dynamics 400B). (B) Immunoblot analysis of HDV RNPs with an antibody specific to the HDAgs (4). Lanes C, L, LM, and S are as described for panel A. Wild-type HDAgs (L and S) and a large HDAg mutant (LM) detected from the immunoprecipitated nuclear lysates are indicated by asterisks. Light chains of the rabbit immunoglobulin were detected in all samples examined.

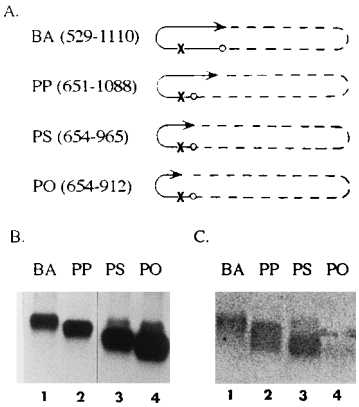

FIG. 8. Northern blot analysis following cotransfection of Huh-7 cells with HDV RNA fragments of various lengths and plasmids encoding small HBsAg and large HDAg. Plasmids representing large HDAg, small HBsAg, and linear HDV RNA fragments from nt 529 to 1110 (lanes 1), 651 to 1088 (lanes 2), 654 to 965 (lanes 3), and 654 to 912 (lanes 4) were used in the cotransfection studies. RNA samples were isolated from total cellular lysates (B) and virus-like particles (C) 4 days posttransfection. The probe used was the antigenomic strand of HDV RNA and was prepared as described in Materials and Methods. The nonspecific signals on the top of the specific band for HDV RNA in lane 4 of panel C are spotty backgrounds.

recent report indicated that HDAgs contain a putative RNA-binding domain near the N terminus (29), a large HDAg with a deletion in the N-terminal domain does not seem to have significant effect on the assembly of HDV RNA (Fig. 5). In addition, a mutation at Leu-115 within the putative helix-loop-helix structure of small HDAg important for in vitro RNA-binding activity and viral replication (7) (Fig. 7B) does not significantly affect the assembly of HDV RNA (Fig. 7B). These results suggest that conformational requirements of small HDAg in the assembly and replication of HDV RNA may be different. HDV genomic RNA appears to replicate through a rolling-circle mechanism upon viral infection, since RNA intermediates longer than the unit-length antigenomic RNA are detected in HDV-infected cells (10, 27). The RNA intermediates are self-cleaved into a unit-length antigenomic RNA that serves as a template for the synthesis of HDV genomic RNA by a similar rolling-circle mechanism (18, 28). Genomic RNA intermediates are then self-cleaved into a unit-length RNA either for a second round of viral replication or for virion assembly (18, 41). In the current study, a monomeric HDV genomic RNA was detected in both in vitro transcription reactions (Fig. 1) and virus-like particles (Fig. 2 and 5), indicating that the HDV RNA can undergo self-cleavage in vitro as well as in transfected cells. Furthermore, it has been demonstrated that viral RNA isolated from HDV particles is present mainly as a circular but partially as a linear form (10). Recently, a study indicated that a circular 348-nt viral RNA could be packaged into virus-like particles (20). Here, we demonstrate that a 312-nt linear HDV RNA containing a putative rod-like structure is sufficient for RNA packaging (Fig. 8). These data suggest that a linear as well as a circular form of HDV genomic RNA can form RNP complexes with HDAgs and can be copackaged with HBsAg for HDV morphogenesis. In addition, a 259-nt linear HDV RNA with mutations at A-900 and C-901 was packaged less efficiently (Fig. 8C, lane 4). This finding implies that RNA sequences from nt 913 to 965 may be important for RNA packaging. Alternatively, mutations at positions 900 and 901 may alter the RNA structure critical for packaging.

It has been proposed that HDV RNA replicates in the nucleus. A host DNA-dependent RNA polymerase II-like enzyme may be involved in the replication of HDV RNA (13, 25). It is possible that the assembly of HDV RNA occurs in the nucleus. However, an HDAg mutant in which the N-terminal nuclear localization signal domain between amino acid residues 35 and 88 (5, 43) had been deleted retained the ability to assemble HDV RNA (Fig. 5A). One possibility for this observation is that the uncleaved HDV RNA with a poly(A) tail was synthesized in the nuclei of transfected cells and transported to the cytoplasm prior to forming RNP complexes with the cytoplasm-localized large HDAg mutant. Alternatively, the large HDAg mutant may interact with HDV RNA when the nuclear envelope is disintegrated during the processes of mitosis. The monomeric HDV RNA has been detected in both the cytoplasm and the nuclei of transfected cells (24), but the exact subcellular locations of morphogenesis of HDV RNA remain to be elucidated.

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