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Small-Form Hepatitis B Surface Antigen Is Sufficient To Help in the Assembly of Hepatitis Delta Virus-Like Particles

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Hepatitis delta virus (HDV) has an envelope composed of large-, middle-, and small-form hepatitis B surface antigens (HBsAgs) provided by the helper hepatitis B virus (HBV). In order to examine the roles of individual HBsAgs in HDV assembly, we constructed plasmids containing each specific HBsAg gene and then cotransfected each plasmid with HDV cDNA into a permissive human hepatoma cell line (HuH-7) to examine the effects on HDV production. Results indicated that the plasmids containing only the HBsAg genes were able to complement HDV cDNA as efficiently as the plasmid containing the complete HBV genome in generating HDV-like particles. Moreover, the small-form HBsAg alone was sufficient for HDV packaging. The particles produced from the cotransfection experiments have density and protein composition characteristics similar to those of naturally occurring HDV. With the electron microscope, they were identified as 36- to 38-nm-diameter particles. It was concluded that only the HBsAgs were able to help in the assembly of HDV-like particles.

Hepatitis delta virus (HDV) is a 36-nm-diameter particle consisting of three components (11, 19). The virion contains a single-stranded, circular RNA genome of approximately 1.7 kb (5, 14, 23). It also contains two virus-specific proteins, the large- and small-form hepatitis delta antigens (HDAgs), within the particles (1). However, the envelope of HDV has been found to be made up, not of the proteins encoded by HDV genome, but of hepatitis B surface antigens (HBsAgs) that are provided by the helper hepatitis B virus (HBV) (1, 25). This last feature indicates that HDV is a defective virus. In natural infection, transmission of HDV actually requires the presence of HBV (18, 19).

The requirement for HBsAgs in the formation of HDV particles is supported by recent experiments. It has been shown that although transfection of HDV cDNA alone into human hepatoma cell lines results in active replication of HDV genome, it does not produce HDV or HDV-like particles (6, 13). HDV virions can be recovered only after cotransfection with the HBV DNA (25). Therefore, the HBV genome contains gene(s) (very likely the HBsAg gene) essential for HDV assembly.

The HBsAgs are composed of three related proteins, namely, the large (L)-, middle (M)-, and small (S)-form HBsAgs (10, 12). Each of the three HBsAgs exists in two forms with different degrees of glycosylation (10, 12). They are encoded by a single long open reading frame in the HBV genome but are produced by three alternative translation initiation sites (Fig. 1). M HBsAg contains S HBsAg plus a pre-S2 region of 55 amino acids at the N terminus (12). L HBsAg contains the entire M HBsAg plus a pre-S1 region of 108 to 119 amino acids at the N terminus (12). In HBV particles (the Dane particles), S HBsAg is the major component and accounts for 60% of the envelope proteins (10). L HBsAg is the second most abundant form and accounts for about 20 to 30% of the total, whereas M HBsAg accounts for less than 20% (10). Corresponding to their relative abundance in the virion, both the L and S HBsAgs have been shown to be essential for HBV formation while M HBsAg is dispensable (2).

The three HBsAgs are also components of the HDV envelope but with proportions different from those in the HBV envelope. S HBsAg is still the major component in the HDV envelope, accounting for about 95%. M HBsAg accounts for about 5%, while L HBsAg accounts for less than 1% (1). This observation suggests that the significance of each HBsAg in the assembly of the HDV virion is probably not the same as in HBV assembly.

Therefore, the role of each HBsAg in the assembly of HDV was examined in the present study. A plasmid containing the L, M, or S HBsAg gene was cotransfected with the HDV cDNA into the permissive HuH-7 cell line (25). The culture medium was collected, and viral particles were purified by centrifugation and were examined with an electron microscope. The nucleic acids and proteins of viral particles were studied by Northern (RNA) blotting and immunoblotting procedures. The results indicated that only the small-form HBsAg is required in the assembly of HDV-like particles.

MATERIALS AND METHODS

Plasmid construction. To prepare a partial HBV genome containing the entire large HBsAg gene and endogenous viral promoters and enhancers (10, 26), an *Apa*I-*Bgl*II fragment was released from the wild-type HBV plasmid (pHBV-48 [24]). The fragment was made blunt by treatment with Klenow fragment and then cloned into the *Sma*I site of pGEM-3Z (Promega, Madison, Wis.). The resulting plasmid, pS1X, contains the entire L HBsAg gene and is able to express all three HBsAgs on transfection. The pS1X plasmid was further digested with *Ban*II to remove most of the pre-S1 region but retains the simian virus 40-like promoter for the pre-S2 transcripts (3). It was then ligated to form the pS2X plasmid that contains the entire M HBsAg gene and is able to express the M and S HBsAgs. Another plasmid,

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pCMV-S, containing a *Bam*HI-*Hpa*I fragment (covering only the S HBsAg gene) under the control of a heterologous cytomegalovirus promoter (22), was also constructed to express only the S HBsAg. Finally, pS1X was digested with *Bst*EII and *Pst*I, made blunt, and then religated to make plasmid pDP-SX. As the pre-S1 region and the N terminus of the pre-S2 region were deleted in this plasmid, pDP-SX can express only S HBsAg. The relevant HBV genome and construction of all the plasmids are depicted in Fig. 1.

Cotransfection to HuH-7 cells. For cotransfection experiments, 10 μg of the plasmid pSVLD3 (containing a tandem trimer of HDV cDNA under the control of the simian virus 40 promoter) and 10 μg of each HBsAg-expressing plasmid were transfected to HuH-7 cells by the calcium phosphate precipitation method (25). The culture medium was collected on days 6, 9, 12, and 15 posttransfection, and the production of HBsAgs was determined by a commercial enzyme-linked immunoassay (IMx; Abbott Laboratories, North Chicago, Ill.) (8).

Isolation of HDV virions by centrifugation. The viral particles in the supernatant were pelleted through a 20% sucrose cushion by spinning for 5 h in an SW40 rotor at 35,000 rpm (model L7; Beckman). The pellet was subjected to equilibrium centrifugation in a discontinuous cesium chloride gradient (from 1.19 to 1.51 g/cm^3) in an SW40 rotor at 35,000 rpm for 24 h. Each 0.5-ml fraction was collected from the bottom of the tube until it was empty. The density of each fraction was determined by a refractometer. Every other fraction was also checked for HDV RNA by Northern blotting.

Electron microscopic study. The cesium chloride gradient fractions containing the most abundant HDV RNA were first diluted with 3 volumes of PBS buffer (0.85% NaCl, 0.01 M phosphate [pH 7.4]). It was then centrifuged in a TL100 rotor for 2 h at 60,000 rpm (model TL100 ultracentrifuge; Beckman) to pellet the viral particles. These concentrated viral particles were examined with an electron microscope after negative staining with 3% uranyl acetate (25).

Radioisotope labeling of viral proteins. HuH-7 cells in a 10-cm-diameter petri dish were transfected with pSVLD3 and a specific HBsAg-expressing plasmid. The culture medium was changed every 3 days after transfection. At day 6 posttransfection, the cells were washed twice with PBS buffer and 2.5 ml of 10% dialyzed fetal bovine serum (Boehringer Mannheim GmbH, Mannheim, Germany) was added to the culture medium. After starvation for 1 h, the ^{35}S-labeled Met residue (525 μCi; Amersham, Amersham, United Kingdom) was added to the cells and incubated for 10 h. The culture medium was collected at days 7 and 10. One milliliter of culture medium was used for immunoprecipitation with mouse anti-HBsAg antibody as the primary antibody and rabbit anti-mouse immunoglobulin as the secondary antibody. The immunoprecipitated HBsAgs were electrophoresed in a 15% polyacrylamide–sodium dodecyl sulfate gel. The gel was then dried and exposed.

Characterization of nucleic acids and proteins. Northern blotting to detect HDV RNA and Western blotting (immunoblotting) to characterize the delta antigens and HBsAgs in the HDV or HDV-like particles were performed as previously described (5, 6).

RESULTS

Plasmids containing the L or M HBsAg gene can efficiently complement HDV cDNA in packaging HDV genome. It has been shown that a partial dimer of HBV, when cotransfected with the HDV cDNA (pSVLD3) into HuH-7 cells, can help produce HDV virions (25). However, as the HDV virion contains no HBV gene products other than the HBsAgs, probably the HBsAg gene alone is sufficient in assisting HDV virion formation. To test this, we constructed three HBsAg-expressing plasmids, pS1X, pS2X, and pCMV-S (Fig. 1B) and examined their effects on HDV packaging by cotransfection with HDV cDNA.

Each HBsAg-expressing plasmid was cotransfected with an equivalent amount of pSVLD3 to HuH-7 cells. Supernatant was collected at day 6 posttransfection, and viral particles were pelleted. RNA was then extracted and analyzed by Northern blotting with a probe specific to HDV. Plasmids pS1X (expressing L, M, and S HBsAg) and pS2X (expressing M and S HBsAg) can help to package the HDV genome (Fig. 2A, lanes 2 and 3) as efficiently as the plasmid containing the entire HBV genome (Fig. 2A, lane 1). These results supported the previous conjecture that HDV needs only HBV envelope proteins for virion formation (1, 13, 25).

Furthermore, we found that only S HBsAg, expressed by the plasmid pCMV-S, could package the HDV genome, although much less efficiently (Fig. 2B, lane 4 versus lane 1).

The inefficiency of pCMV-S in assisting HDV packaging may be due to low-level production of HBsAg. The inefficiency of pCMV-S in helping to package the HDV genome could be due to a defective characteristic of S HBsAg. However, the possibility of inadequate expression of the pCMV-S plasmid has to be addressed first. Thus, the culture medium was collected at days 3, 6, 9, 12, and 15 posttransfection and the amount of secreted HBsAgs was determined by an automatic enzyme-linked immunoassay (8).

As shown in Fig. 3, the amount of HBsAgs produced by pS1X and pS2X was as high as that by the wild-type HBV plasmid and persisted for 15 days. However, that generated by plasmid pCMV-S was not only 10-fold less but also declined rapidly before day 6, a time when HDV virions start to release from transfected cells (25). These results suggest that poor expression of HBsAg is an important factor related to the inefficiency of pCMV-S in packaging the HDV genome.

A plasmid efficiently expressing only S HBsAg can help in the HDV packaging. In order to boost the expression of S HBsAg, we constructed plasmid pDP-SX which was derived from pS1X by deleting the entire pre-S1 region and the N terminus of the pre-S2 region (Fig. 1). On transfection into HuH-7 cells, pDP-SX caused levels of HBsAg production as high and long-lasting as those caused by pS1X (Fig. 3). When normalized to the amount of HBsAg production (Fig. 4B), it was found that the packaging of HDV RNA was done as effectively as that of the pS1X or pS2X plasmid (Fig. 4A). The results indicated that the S HBsAg alone was sufficient in assisting HDV genome packaging. (In this experiment, the culture medium was used for immunoprecipitation of HBsAg; therefore, only S and M HBsAgs were demonstrated. The less abundant L-form HBsAg could be detected only after further enrichment of HDV-like particles by isopycnic centrifugation as shown in Fig. 6).

In addition, total RNA was extracted from those immunoprecipitated HBsAg-containing particles and then subjected to Northern analysis. As shown in Fig. 4C, HDV RNA was detected in the three kinds of immunoprecipitated HBsAg-containing particles (lanes 2, 3, and 4). The results indicated that HDV RNA was indeed copackaged with HBsAg.

Characterization of the HDV-like particles produced in the three cotransfection experiments. The successful packaging

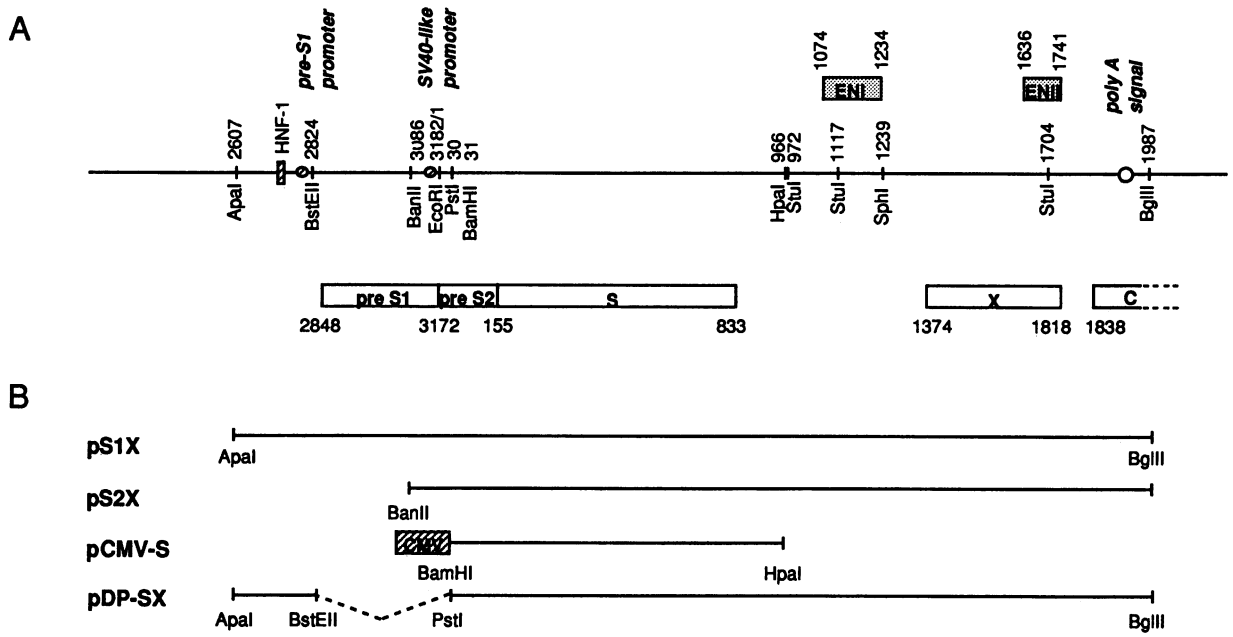

FIG. 1. Diagram showing the organization of HBV genome and construction of plasmids. (A) HBV genome. The restriction enzyme sites and their nucleotide numbers on the HBV genome are shown at the top of the panel (9). The viral genes are indicated at the bottom of the panel. Viral transcriptional regulatory element abbreviations: HNF-1, the site for hepatic nuclear factor I binding; ENI, enhancer I; ENII, enhancer II (10, 26). (B) Construction of HBsAg-expressing plasmids. Plasmid pS1X was constructed by inserting the *Apa*I-*Bgl*II fragment of HBV genome into the *Sma*I site of pGEM-3Z. pS2X contained the *Ban*II-*Bgl*II fragment. pCMV-S was constructed by putting the *Bam*HI-*Hpa*I fragment under the cytomegalovirus promoter. pDP-SX was made by deleting the *Bst*EII-*Pst*I fragment from pS1X.

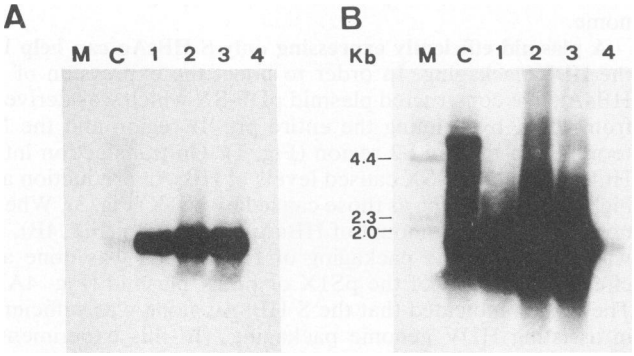

FIG. 2. HBV surface antigens can help package HDV genome. The effects of three HBsAg-expressing plasmids on HDV packaging were examined by cotransfection with pSVLD3 to HuH-7 cells. The culture supernatant of cotransfected HuH-7 cells was pelleted, and RNA was extracted for Northern blotting analysis with a probe specific for HDV RNA. Lane 1, positive control showing the result of hybridization of RNA resulting from cotransfection of pSVLD3 and a plasmid containing the entire HBV genome, pHBV-48; lanes 2 to 4, the hybridization results of pellets from cells cotransfected with pSVLD3 and pS1X (lane 2), pSVLD3 and pS2X (lane 3), and pSVLD3 and pCMV-S (lane 4); lane M, lambda *Hin*dIII fragments (size markers); lane C, hybridization result of the total RNA from stable HepG2 cells capable of continuous HDV replication (6). The autoradiograph in panel A was exposed for 2 h, while that in panel B was exposed for 40 h.

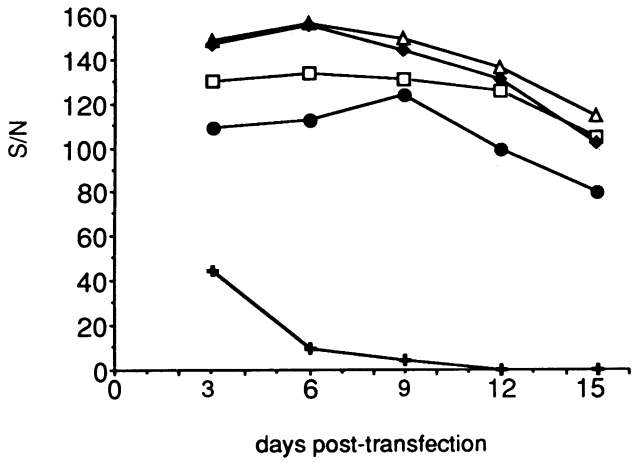

FIG. 3. Temporal profile of HBsAg production directed by the five HBsAg-expressing plasmids cotransfected with pSVLD3. To determine the amount and time course of HBsAg production directed by these plasmids, the culture medium was collected at days 3, 6, 9, 12, and 15 posttransfection. A 0.2-ml aliquot was checked by an automatic enzyme-linked immunosorbent assay reader to determine HBsAg production and was expressed as S/N ratio (sample versus negative control). Each point represents the mean from at least two independent experiments. Plasmid pCMV-S produced much less HBsAg and did so in a transient manner. Symbols: △, pSVLD3 and pS2X; ◆, pSVLD3 and pDP-SX; □, pSVLD3 and pS1X; ●, pSVLD3 and pHBV-48; +, pSVLD3 and pCMV-S.

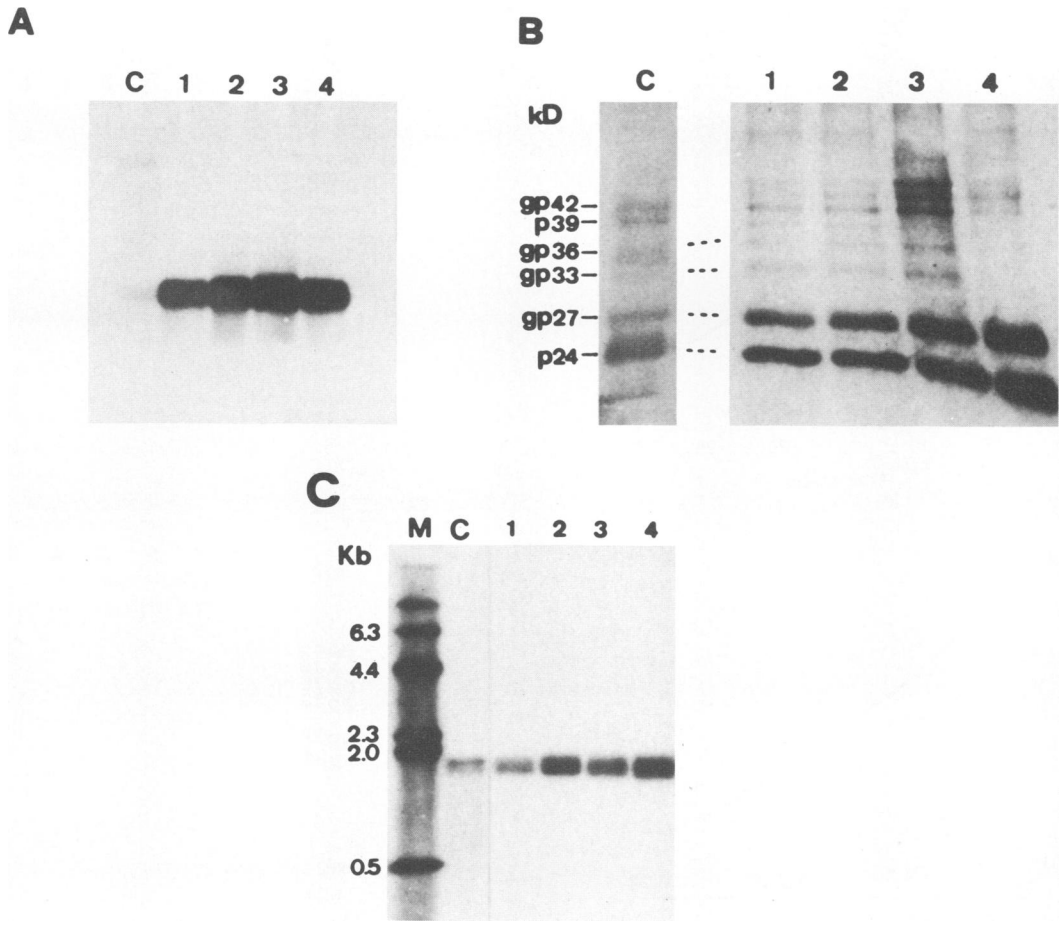

FIG. 4. The S HBsAg is sufficient in helping the HDV genome package. Ten-milliliter samples of supernatant collected at day 6 were centrifuged to precipitate the pellets. (A) Northern analysis of HDV RNA from pellets produced by HuH-7 cells cotransfected with pSVLD3 and pHBV-48 (lane 1); pSVLD3 and pS1X (lane 2), pSVLD3 and pS2X (lane 3), or pSVLD3 and pDP-SX (lane 4). Equivalent amounts of HDV RNA were recovered. (B) Immunoprecipitation to demonstrate a similar level of ^{35}S-labeled HBsAg in the culture medium of these transfected cells. Lane C shows the contents of HBsAgs in Dane particles as a control. gp42 and p39 are the glycosylated and unglycosylated forms of L HBsAg, respectively; gp36 and gp33 are the two forms of M HBsAg differing in the degree of glycosylation; gp27 and p24 are the glycosylated and unglycosylated forms of S HBsAg, respectively (12). (C) Demonstration of HDV RNA in immunoprecipitated HBsAg-containing particles. Following precipitation of the three kinds of particles from the culture medium with antibody against HBsAg, total RNA was extracted and then subjected to Northern analysis for HDV RNA. Lane M shows the lambda *Hin*dIII fragments as markers. Lane C is a positive control made by immunoprecipitating HDV virions from a patient's serum. HDV RNA is detected. Lanes 1 to 4 in panels B and C are as described for panel A.

of HDV genome in the cotransfection experiments strongly suggested the production of HDV or HDV-like particles. Therefore, further characterization was undertaken. The pellets recovered from the three cotransfection experiments were subjected to isopycnic centrifugation in a CsCl gradient. The fractions containing HDV RNA were then identified by Northern blotting.

After isopycnic centrifugation, the peaks of HDV RNA of the three kinds of particles were localized to fractions of density from 1.24 to 1.25 g/cm^3 (Fig. 5). Therefore, in spite of the different compositions of the viral envelope proteins, the three kinds of particles had the same density as that of the wild-type HDV (7, 19).

Furthermore, the proteins of viral particles in these peak fractions were analyzed for the presence of delta antigens and HBsAgs. It was found that both the large and small forms of delta antigens were included in these particles (Fig. 6A, bottom gel). The composition of envelope proteins of three kinds of particles was also found to be as expected from the plasmid constructs. There were six species of HBsAg proteins (corresponding to L, M, and S HBsAg with different levels of glycosylation) in particles produced by cotransfection of pS1X and pSVLD3 (Fig. 6A, top gel). Four species of HBsAgs were found in the particles produced by cotransfection with pS2X and pSVLD3 (Fig. 6B, top gel), while only two species were found in these by cotransfection with pDP-SX and pSVLD3 (Fig. 6C, top gel).

In summary, these results indicated that the physicobiochemical characteristics of these particles were similar to those of wild-type HDV virions (1, 17, 19).

Electron microscopy of the HDV or HDV-like particles. Finally, the particles from the three peak fractions were examined with an electron microscope. In that produced from cotransfection with pS1X and pSVLD3, spherical particles with a mean diameter of 38 nm were observed among some 22-nm-diameter spherical and filamentous HBsAg particles (Fig. 7A). The sizes and shapes of these 38-nm-diameter particles were similar to those of naturally

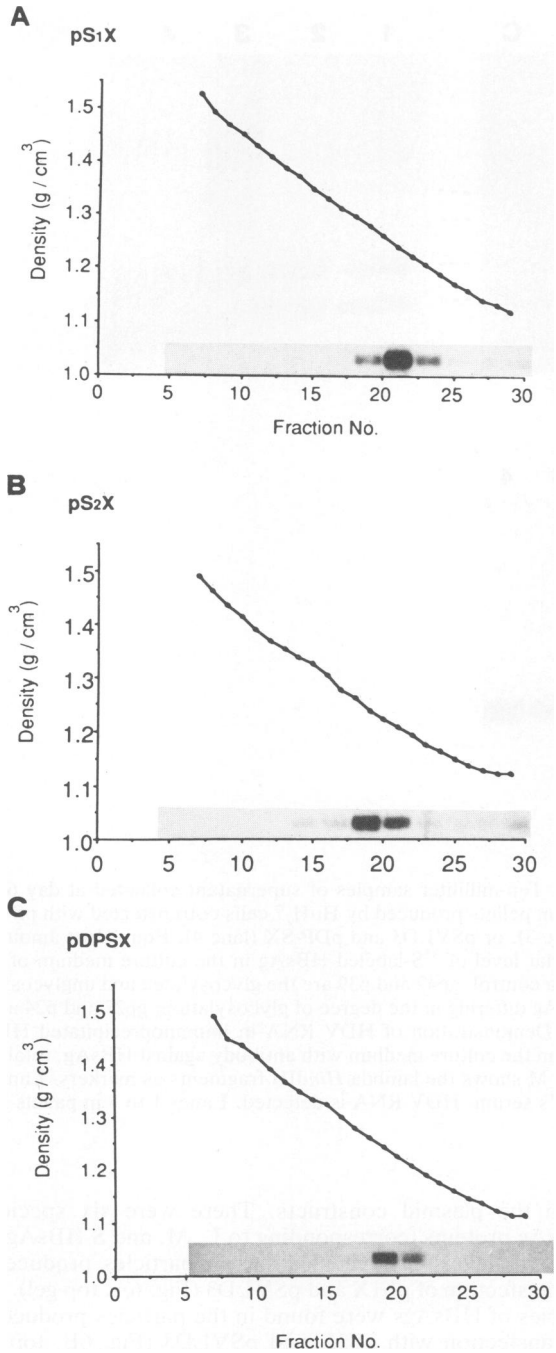

FIG. 5. Densities of the three kinds of HDV-like particles in cesium chloride gradient. The pellet recovered from the culture medium of HuH-7 cells cotransfected with pSVLD3 and pS1X was subjected to isopycnic banding in a discontinuous CsCl gradient as described previously (25). About 30 fractions were collected, and their densities were determined with a refractometer. Nucleic acids from every other fraction were extracted and examined for HDV RNA by Northern blotting (the inset gels). The plot shows the major peak of HDV RNA at a density of about 1.24 g/cm^3 (A). Similar studies were conducted for pellets produced from HuH-7 cells cotransfected with pSVLD3 and pS2X (B) or with pSVLD3 and pDP-SX (C).

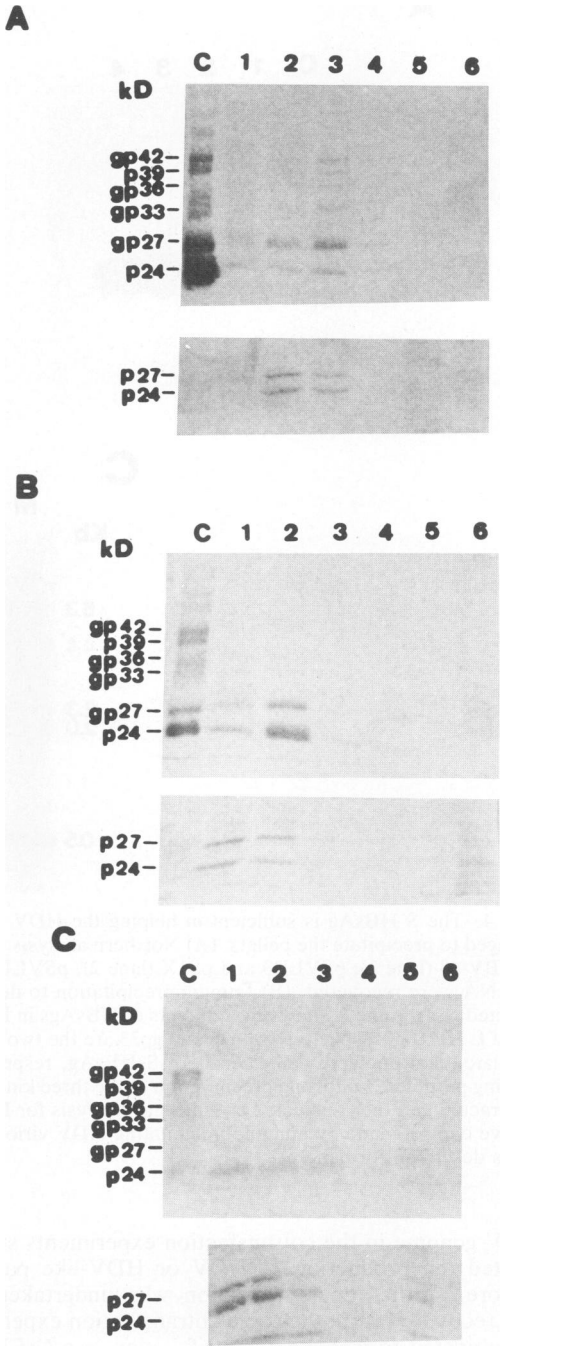

FIG. 6. Protein components of the three kinds of HDV-like particles. The CsCl fractions from around the fraction containing the peak level of HDV RNA were pelleted and analyzed for the presence of HBsAgs and HDAgs by immunoblotting. (A) The HDV-like particles recovered from such fractions (produced by HuH-7 cells cotransfected with pSVLD3 and pS1X) were studied by immunoblotting with antibodies specific for HBsAgs. Wild-type HBV purified from patients was used as a control and six species of HBsAg are shown (lane C). Western blotting with an antibody specific for HDAg was also performed for these fractions (lanes 1 to 6 contain samples from fractions 19, 21, 23, 25, 27, and 29, respectively); these results are shown on the bottom gel in panel A. Both the large- and small-form HDAg (p27 and p24) were found. The same studies were conducted on the particles recovered from HuH-7 cells cotransfected with pSVLD3 and pS2X (B) or with pSVLD3 and pDP-SX (C).

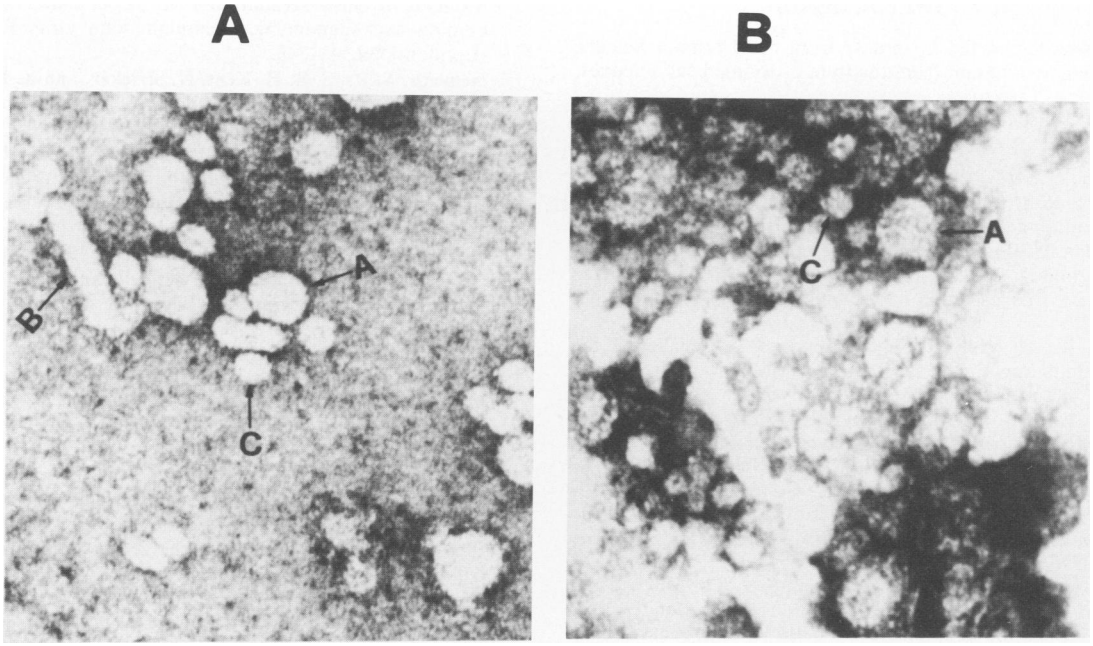

FIG. 7. Electron microscopic images of the HDV-like particles. HDV-like particles obtained from the CsCl fractions with peak HDV RNA were concentrated by ultracentrifugation. The particles were negatively stained with 3% uranyl acetate and then examined with an electron microscope. (A) Viral particles produced from HuH-7 cells cotransfected with pSVLD3 and pS1X. Magnification, ×257,500. Arrow A designates the HDV-like particles with a mean diameter of 38 nm (range of 31 to 39 nm). Arrows B and C indicate the filamentous HBsAg particles with a mean diameter of 22 nm and the spherical HBsAg particle with a mean diameter of 21 nm, respectively. (B) HDV-like particles produced from HuH-7 cells cotransfected with pSVLD3 and pDP-SX. Arrow A designates the HDV-like particle with a mean diameter of 36 nm (31 to 43 nm). Arrow C indicates the spherical-form HBsAg particle with a mean diameter of 21 nm. Magnification, ×257,500.

occurring HDV virions (17, 19). Viral particles with the same characteristics were found in those fractions obtained from cotransfection with pS2X and pSVLD3 (not shown) or pDP-SX and pSVLD3 (Fig. 7B). Consistent with the previous results, it was noted that the filamentous HBsAg particles were present only in the fractions produced by cotransfection with pS1X and pSVLD3 (12).

DISCUSSION

In this study, we demonstrated that the sole helper function of HBV was to provide the envelope proteins in HDV virion assembly. In addition, it was found that the small-form HBsAg was sufficient in helping formation of HDV-like virions. Our study confirmed the previous proposal suggesting that HDV requires HBV surface proteins for transmission and resolved the close relationship between the two hepatitis viruses (13, 20).

The three kinds of HDV-like particles appear to be very similar to the naturally occurring HDV (11, 19). However, it is not known whether they are infectious. It is therefore very important to inoculate them into chimpanzees or woodchucks carrying the helper viruses (17, 19–21) and observe the outcome. Moreover, as the three kinds of particles have different compositions of HBsAgs, the infection experiments could provide clear information about the significance of individual HBsAgs in HDV infection. For HBV infection, either the pre-S1 or pre-S2 region has been shown to bind the putative virus receptors and has been implicated in virus entry (15, 16). It should be interesting to see whether the same domain is used by HDV for infection.

Results of our study also indicated that the assembly of HDV is quite different from that of HBV. For instance, L HBsAg, which is essential for HBV formation, is not required for HDV formation (2). In addition, these HDV-like particles also have an envelope protein composition very similar to that of the spherical or filamentous-form HBsAg particles which do not contain the HBV genome (1, 12). This result suggested that the HDV proteins (the large- and small-form HDAgs) and RNA could be packaged into such particles efficiently. Because it is well-known that in HBV infection defective HBsAg particles are 10- to 1,000-fold more abundant than Dane particles (10, 12), using this packaging pathway certainly provides an advantage for HDV. Indeed in acute infection, the HDV infectivity titer frequently reached 10^9 to 10^{10}/ml, a level much higher than that of HBV (18).

Our study indicated that only S HBsAg was sufficient to help assemble HDV-like particles. However, which domain in the S HBsAg actually participates in the morphogenesis is not yet known. On the basis of results from previous studies on the topology of HBsAg, both the N and C termini of S HBsAg have been shown to be locked in the membrane of endoplasmic reticulum (7, 10). Therefore, they are unlikely to be directly involved. Perhaps the central domain of HBsAg facing the lumen of endoplasmic reticulum is more important for HDV assembly. Further deletion analysis of pDPSX should be able to answer this question. In addition, we have recently shown that the large form of HDAg is also crucial in HDV assembly (4). Therefore, the interactions between the small HBsAg and the large HDAg may be the key element for HDV virion formation, an area worthy of further investigation.

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