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Role of the Large Hepatitis B Virus Envelope Protein in Infectivity of the Hepatitis Delta Virion

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The hepatitis delta virus (HDV) is coated with large (L), middle (M), and small (S) envelope proteins encoded by coinfecting hepatitis B virus (HBV). To study the role of the HBV envelope proteins in the assembly and infectivity of HDV, we produced three types of recombinant particles in Huh7 cells by transfection with HBV DNA and HDV cDNA: (i) particles with an envelope containing the S HBV envelope protein only, (ii) particles with an envelope containing S and M proteins, and (iii) particles with an envelope containing S, M, and L proteins. Although the resulting S-, SM-, and SML-HDV particles contained both hepatitis delta antigen and HDV RNA, only particles coated with all three envelope proteins (SML) showed evidence of infectivity in an in vitro culture system susceptible to HDV infection. We concluded that the L HBV envelope protein, and more specifically the pre-S1 domain, is important for infectivity of HDV particles and that the M protein, which has been reported to bear a site for binding to polymerized albumin in the pre-S2 domain, is not sufficient for infectivity. Our data also show that the helper HBV is not required for initiation of HDV infection. The mechanism by which the L protein may affect HDV infectivity is discussed herein.

Hepatitis delta virus (HDV) was initially described as a subviral agent that relies on the presence of hepatitis B virus (HBV) for propagation (32, 33). Subsequent analysis of the viral particle revealed that HBV plays at least one essential role in the life cycle of HDV by providing the proteins that constitute the virion envelope (2, 3, 33). The 36-nm HDV particle consists therefore of an outer envelope of HBV origin and an inner ribonucleoprotein complex made of a circular single-stranded RNA genome and two HDV-encoded proteins that bear the hepatitis delta antigen (HDAg). The HDV genome does not have the coding capacity for its own envelope proteins but codes for the HDAg proteins, the only HDV-encoded proteins known to date (1, 17, 43, 44).

The envelope of HBV consists of lipids and three unglycosylated or glycosylated viral proteins translated from a single open reading frame on the HBV genome (11). The three proteins, designated small (S), middle (M), and large (L), are translated from three in-phase start codons to a common stop codon. The smallest gene encodes the S protein (p24/gp27), which bears the hepatitis B surface antigen (HBsAg). The M protein (gp33/gp36), which bears pre-S2Ag and HBsAg, is encoded by a larger gene containing the pre-S2 and S regions, whereas the L protein (p39/gp42), which bears pre-S1Ag, pre-S2Ag, and HBsAg, is encoded by the largest gene containing the pre-S1, pre-S2, and S regions. Mature HBV (Dane) particles (7) are composed of approximately equimolar proportions of all three species, whereas 22-nm-diameter subviral particles present in large numbers in an infectious serum are composed primarily of the S protein with low amounts of the M protein (11).

Studies on the role of the envelope proteins in the assembly of mature HBV particles have demonstrated the requirement for the L and S proteins (4, 41). Other studies indicate that the pre-S1 and pre-S2 domains may be involved in the attachment of HBV to the hepatocyte (8, 13, 20, 25, 28–30). The envelope of mature HBV particles contains higher amounts of pre-S1 polypeptides than the envelope of empty subviral particles. It is at least partially exposed at the surface of the virion (13, 18) and may contain a site for direct binding of HBV to a receptor on the hepatocyte (8, 20, 25, 28–30). The pre-S2 domain of the M protein is also exposed at the surface of the HBV envelope and contains a site for binding to polymerized albumin (21). Whether this property can elicit indirect binding of HBV to the hepatocyte membrane remains uncertain.

The envelope of HDV particles includes also lipids and all three glycosylated or unglycosylated HBV envelope proteins (2, 3, 12), but in comparison with the envelope of HBV particles, there are fewer of the L (1%) and the M (5%) proteins. As in HBV particles, the pre-S1 and pre-S2 polypeptides are exposed at the surface of HDV particles as evidenced by the ability of anti-pre-S1 and anti-pre-S2 antibodies to neutralize infection in vitro (37). Therefore, the L and M proteins also may have an important role in recognizing the HDV receptor on the cell membrane, but in contrast with HBV, these proteins are not required to assemble a genome-containing HDV particle (34, 42). These data suggest that HBV envelope proteins interact with each other for assembly of HDV particles in a manner different from that required to assemble Dane particles but similar to that used to assemble empty 22-nm HBV subviral particles.

In the present study, we addressed the role of the HBV envelope proteins in the assembly and infectivity of HDV particles and, specifically, the function of the L protein. This analysis was performed in an in vitro model system in which particles coated with envelopes of different compositions were produced in Huh7 cells by transfection with HBV DNA and HDV cDNA (37, 45) and assayed for infectivity in primary cultures of chimpanzee hepatocytes. Chimpanzee hepatocyte cultures are required because of the resistance of the Huh7 cell line to infection. Our results indicate that the L envelope protein plays a crucial role in HDV infectivity.

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MATERIALS AND METHODS

Plasmid constructions. An *Xho*I (nucleotide [nt] 130)-to-*Bgl*II (nt 1987) fragment of HBV DNA *ayw* subtype was inserted between the *Xho*I and *Bam*HI sites of the pMT-neo plasmid (27). The resulting recombinant plasmid, pMTS-neo, contains the S gene for expression of the S HBV envelope protein. For the construction of plasmid pMTSM-neo, a *Bam*HI (nt 2907)-to-*Bgl*II (nt 1987) fragment of HBV DNA subtype *ayw* was inserted in the *Bam*HI site of the pMT-neo plasmid. This recombinant plasmid contains the pre-S2–S gene and is used to express the S and M HBV envelope proteins. Plasmid pMTSML-neo contains a *Bgl*II (nt 2840)-to-*Bgl*II (nt 1987) fragment of HBV DNA subtype *ayw* inserted in the *Bam*HI site of the pMT-neo plasmid. Plasmid pMTSML-neo contains the pre-S1–preS2–S gene and can direct the expression of the S, M, and L proteins. All three plasmids contain the mouse metallothionein (MT) promoter and HBV polyadenylation signals for expression of HBV envelope proteins. They also include a transcription unit for the expression of the neomycin resistance gene.

The recombinant plasmid pSVLD3 contains a head-to-tail trimer of full-length HDV cDNA for expression of HDV genomic RNA under the control of the simian virus 40 late promoter (16). The recombinant plasmid pFC80 (F. Chisari) contains a head-to-tail tetramer of full-length HBV DNA *ayw* inserted in the *Eco*RI site of pBR322. Transfection of Huh7 cells with this plasmid results in replication of HBV DNA and production of Dane-like particles.

Transfection of Huh7 cells. For expression of HBV envelope proteins in stable cell lines, the Huh7 cells were transfected with pMTS-neo, pMTSM-neo, or pMTSML-neo plasmid as described earlier (38). After transfection, cells were maintained in medium containing the neomycin analog G418 (GIBCO BRL, Gaithersburg, Md.). Neomycin-resistant clones were isolated and tested for expression of HBV envelope proteins. Positive clones expressing the S, SM, and SML HBV proteins were expanded and termed Huh7-S, Huh7-SM, and Huh7-SML, respectively.

For production of HDV particles, Huh7-S, -SM, and -SML cells were propagated in Dulbecco modified Eagle medium-F12 medium supplemented with 10% fetal bovine serum. Cells were seeded at 4×10^5 cells per 60-mm-diameter petri dish and transfected with 10 μg of HDV recombinant plasmid pSVLD3 (16) at 24 h after seeding by the procedure of Chen and Okayama (6). Cells were exposed to the DNA-phosphate precipitate for 5 h and washed with phosphate-buffered saline (PBS) before incubation in culture medium. The culture medium was harvested every 3 days thereafter for purification of viral particles.

Preparation and characterization of virus-like particles produced in Huh7 cells. Culture medium harvested on days 6 and 9 after transfection was used for purification of viral particles. Culture medium was clarified by centrifugation at 5,000 × *g* for 1 h at 4°C and concentrated 20 times in a Centricon 100 microconcentrator. The concentrate was resuspended in serum-free medium, resulting in a 5× concentration that served as the inoculum in infectivity assays. For analysis of HDV particles, clarified medium was then layered onto a 5-ml 20% sucrose cushion in PBS and subjected to centrifugation at 25,000 rpm at 4°C for 16 h in an SW28 rotor. The pellet was resuspended in PBS, loaded on a 10 to 50% (wt/vol) CsCl gradient in PBS, and subjected to centrifugation at 38,000 rpm in an SW41 rotor for 18 h at 4°C. Fractions were collected from the bottom of the tube, and the density was determined by measurement of the refractive index. Aliquots of each fraction were used for detection of HDV RNA by Northern (RNA) blot analysis and detection of HBsAg by an enzyme-linked immunoassay (Auzyme EIA; Abbott Laboratories, North Chicago, Ill.).

Detection of HBV envelope proteins by immunoblot analysis. Particles sedimented from the culture medium were disrupted in 100 μl of buffer containing 2% sodium dodecyl sulfate and 2% β-mercaptoethanol, heated at 100°C for 5 min, and analyzed by immunoblotting as described previously (19) with either human anti-HDAg antibodies (provided by F. Negro from J. Gerin's laboratory) or rabbit anti-HBsAg antibodies (Calbiochem, San Diego, Calif.).

Immunoprecipitation of HDV particles for detection of HDV RNA. Culture medium harvested on days 6 and 9 after transfection was incubated at 4°C overnight in the presence of monoclonal antibodies specific for HIV gp120 envelope protein (provided by R. Attanasio) as a negative control or monoclonal antibodies specific for the S (provided by R. C. Kennedy) or the pre-S1 and pre-S2 domains of the HBV envelope proteins (14). The virus-antibody complex was then bound to protein G-Sepharose (Sigma Chemical Co., St. Louis, Mo.) by incubation at 4°C for 2 h. Beads were washed three times with PBS, and RNA was extracted from the beads for detection of HDV RNA sequences by RNA blot hybridization.

Primary cultures of chimpanzee hepatocytes. Hepatocytes were isolated from a wedge biopsy obtained from chimpanzees. These animals were free of any HDV markers including HDV RNA and anti-HDAg antibodies in the serum and had no history of HDV infection. The procedures utilized for the isolation and culture of primary hepatocytes in a serum-free medium formulation have been described previously (15).

In vitro infections. Cells were exposed to HDV for at least 12 h on day 3 after seeding. Inocula consisted of particles harvested from transfected Huh7 cells as described above. Particles concentrated from one 60-mm petri dish of transfected Huh7 cells were resuspended in 1 ml of serum-free medium and added to a 22-mm-diameter well containing 10^6 hepatocytes. By measurement of HDV RNA extracted from the inoculum and comparison to a known amount of HDV cDNA, we estimated the concentration of HDV genomes in the inoculum. After exposure, cells were washed and incubated in 1 ml of fresh serum-free medium. Cells were harvested every 3 days thereafter for detection of intracellular HDV RNA by RNA blot hybridization.

Detection of HDV RNA. Total cellular RNA was prepared by disrupting the cells in 6 M guanidinium isothiocyanate–5 mM sodium citrate (pH 7.0)–0.1 mM β-mercaptoethanol–0.5% sarcosyl and by centrifugation through a CsCl cushion as described previously (38). HDV RNA from culture medium was purified by incubation in 50 mM Tris-HCl (pH 7.8)–200 mM NaCl–20 mM Na_2EDTA–2% SDS–1 mg of proteinase K per ml–2 mM vanadyl ribonucleoside complex for 2 h and phenol extraction as described previously (39). RNA samples were subjected to electrophoresis through a 1.5% agarose–2.2 M formaldehyde gel and then transferred to nitrocellulose for hybridization to an HDV-specific RNA probe. Strand-specific ^{32}P-labeled riboprobes were synthesized to detect either genomic or antigenomic HDV RNA. Labeled full-length HDV RNA probes were derived from in vitro transcription with the SP6 or T7 RNA polymerase.

For extraction of HDV RNA from immunoprecipitated particles, beads were washed four times in cold PBS and HDV RNA was extracted for RNA blot hybridization.

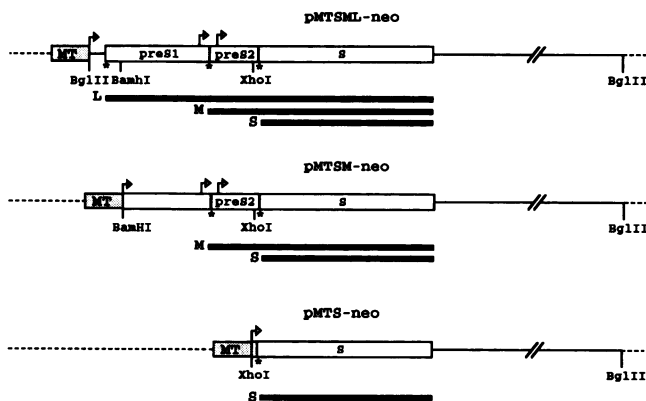

FIG. 1. Recombinant plasmids pMTSML-neo, pMTSM-neo, and pMTS-neo direct the synthesis of the SML, SM, and S HBV envelope proteins, respectively. The S, M, and L proteins are depicted by horizontal dark bars. Plain lines indicate HBV sequences; dashed lines, plasmid sequences; open boxes, HBV envelope open reading frame divided in pre-S1, pre-S2, and S domains; and shaded boxes, MT promoter. Stars indicate AUG start codons for the L, M, and S genes. Initiation of transcription is designated by arrows.

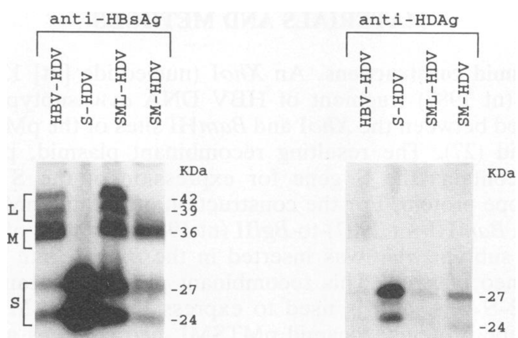

FIG. 2. Immunoblot analysis of HBsAg (left panel)- and HDAg (right panel)-related proteins extracted from culture medium of Huh7 cells transfected with pFC80 and pSVLD3 (HBV-HDV) and Huh7-S, Huh7-SML, and Huh7-SM cells after transfection with plasmid pSVLD3 (S-HDV, SML-HDV and SM-HDV, respectively). Particles were sedimented from 10 ml (HBV-HDV, S, and SM) or 30 ml (SML) of culture medium as described in Materials and Methods and were disrupted in 100 μl of buffer containing 2% SDS and 2% β-mercaptoethanol and heated at 100°C for 5 min. Proteins were separated on a 12% acrylamide gel, transferred to nylon membrane, and analyzed as described previously (19) with either rabbit anti-HBsAg or human anti-HDAg antibodies. The molecular masses of the L, M, and S HBV envelope proteins and the HDAg proteins are indicated at the right side of each autoradiogram. The size markers were prestained proteins (Bethesda Research Laboratories).

RESULTS

Isolation of Huh7-derived cell lines for production of HBV envelope proteins. Expression vectors able to direct the production of the S, SM, or SML HBV envelope proteins and the neomycin resistance gene were designed (Fig. 1). Plasmid pMTS-neo contains the S gene and directs expression of S mRNA controlled by the MT promoter and the HBV polyadenylation signal. Plasmid pMTSM-neo contains the pre-S2–S gene. Expression of M mRNA can be driven from both the MT promoter and the HBV S promoter, whereas expression of S mRNA is controlled by the S HBV promoter. Plasmid pMTSML-neo contains the pre-S1–pre-S2–S gene and allows for expression of L mRNA from the MT promoter and M and S mRNA from the S HBV promoters.

After transfection, single-cell colonies were isolated and grown independently in neomycin-containing medium. Neomycin-resistant clones were screened for the expression of S, SM, or SML proteins. HBV envelope proteins were identified in culture medium after sedimentation of particles and immunoblot analysis with an anti-HBsAg antibody. Positive clones, designated Huh7S, Huh7SM, and Huh7 SML, expressing the S, SM, and SML proteins, respectively, were expanded and used for production of HDV-like particles by transfection with pSVLD3.

The amount of HBsAg released in the culture medium of the Huh7SML cells was significantly lower than that of the Huh7S or Huh7SM cells because of overexpression of the L protein, which has been shown to suppress the secretion of S and M envelope proteins.

Characterization of HDV-like particles produced in vitro. For production of HDV-like particles, the Huh7S, Huh7SM, and Huh7SML cells were transfected with pSVLD3. The resulting HDV-like particles were designated S-HDV, SM-HDV and SML-HDV, respectively. Particles harvested on days 6 and 9 after transfection were then sedimented, and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an anti-HBSAg antibody (Fig. 2). The S proteins appeared as 24- and 27-kDa polypeptides, the M proteins were detected at a molecular mass of 35 to 36 kDa, and the L proteins were detected at 39 and 42 kDa. As expected, only the S proteins were detected in particles released in the culture medium of Huh7S cells. Huh7SM cells produced particles with an envelope containing the S and M proteins, whereas Huh7SML produced particles with an envelope containing the S, M, and L proteins. Particles designated HBV-HDV produced by Huh7 cells after cotransfection with pSVLD3 and pFC80 were used as a control. The results of this immunoblot analysis reflect the protein composition of all particles released in the culture medium of transfected cells including empty 22-nm HBV particles which are produced by the S, SM, and SML cells before transfection with pSVLD3.

Sedimented particles were also analyzed for the presence of HDAg polypeptides by using a human anti-HDAg antibody. HDAg proteins of 24 and 27 kDa were detected (Fig. 2) in all three types of particles. The ratio of p24 to p27 was similar in S-HDV, SM-HDV, SML-HDV, and HBV-HDV particles as estimated by scanning the autoradiogram with a laser densitometer.

For further characterization, HDV-like particles sedimented from the culture medium were subjected to centrifugation on a CsCl gradient (Fig. 3). Fractions were collected from the bottom of the tube, and each fraction was analyzed for density and the presence of HBsAg and HDV RNA. HBsAg and HDV genomic RNAs were detected at 1.24 and 1.20 g/cm^3 density (fractions 5 and 6) for S-, SM-, SML-, or HBV-HDV particles. These results indicated that HDV RNA of genomic size and polarity was detected in particles of densities characteristic of serum-derived HDV particles (fractions 6 and 7 contain also a high amount of empty HBV particles produced by each S, SM, or SML cell line before transfection with pSVLD3). We confirmed here that, as described previously (34, 42), the S HBV envelope protein is sufficient for the assembly of an HDV-like particle that contains genomic HDV RNA.

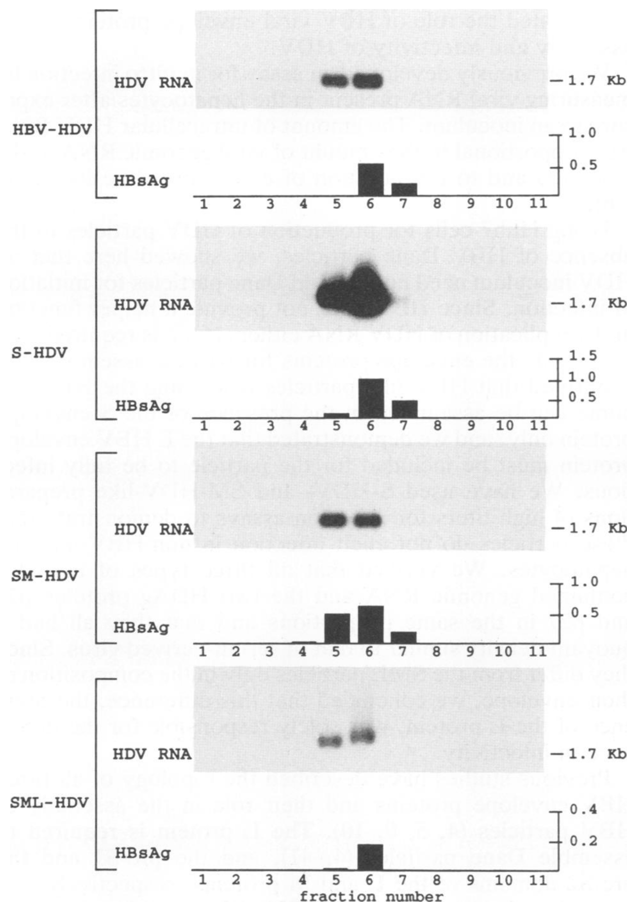

FIG. 3. Analysis of particles derived from culture medium of Huh7 cells transfected with pFC80 and pSVLD3 (HBV-HDV), and Huh7-S, Huh7-SML, and Huh7-SM cells after transfection with plasmid pSVLD3 (S-HDV, SML-HDV, and SM-HDV, respectively) by centrifugation on a CsCl gradient. Particles were sedimented from 30 ml of culture medium as described in Materials and Methods. The pellet was resuspended in PBS, loaded on a 10 to 50% (wt/vol) CsCl gradient in PBS, and subjected to centrifugation for 18 h at 38,000 rpm in an SW41 rotor at 4°C. Fractions were collected from the bottom of the tube, and one-third of each fraction was used for RNA extraction and one-third for detection of HBsAg by enzyme-linked immunoassay. RNA was isolated and separated on a 1.5% agarose–2.2 M formaldehyde gel and analyzed for the presence of genomic HDV RNA after transfer to nitrocellulose and hybridization to a ^{32}P-labeled HDV-specific RNA probe. After hybridization, filters were washed, dried, and autoradiographed at −70°C for 12 h (HBV-HDV, S-HDV, and SM-HDV) or 48 h (SML-HDV) with an intensifying screen. The numbering (1 to 11) of each fraction is indicated. Fractions 5 and 6 correspond to 1.24 and 1.20 g/cm^3 density, respectively. The size of HDV genomic RNA (in kilobases) is indicated. The amount of HBsAg is expressed as A_{492}.

To verify that HDV RNA-containing particles produced in Huh7S, Huh7SM, or Huh7SML cells were indeed coated with the S, SM, and SML envelope proteins, respectively, culture medium from each cell line was incubated in the presence of monoclonal antibodies specific for the S, pre-S2, or pre-S1 domain of the HBV envelope proteins. Immunoprecipitated particles were then analyzed for the presence of genomic HDV RNA by Northern (RNA) blot. As expected, HDV RNA-containing particles derived from the Huh7S cells were immunoprecipitated with anti-S antibodies only

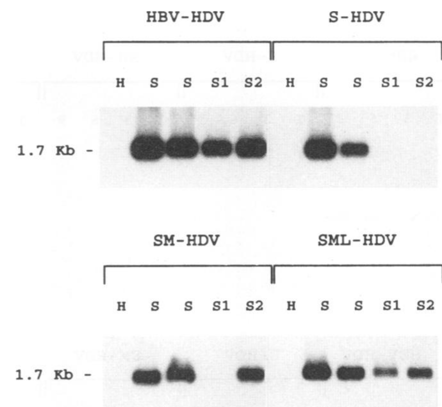

FIG. 4. RNA blot hybridization analysis of HDV RNA extracted from HDV particles immunoprecipitated with monoclonal anti-HIV gp120 (lane H), anti-S 1.2 (lane S), anti-S 2.1 (lane S), anti-pre-S1 M18/7 (lane S1), and anti-pre-S2 Q19/10 (lane S2). HBV-HDV particles were produced by cotransfection of Huh7 cells with pFC80 and pSVLD3. HDV-like particles S, SM, and SML were produced by transfection of the Huh7-S, Huh7-SM, and Huh7-SML cells, respectively, with pSVLD3. RNA was isolated from the beads and separated on a 1.5% agarose–2.2 M formaldehyde gel and analyzed for the presence of genomic HDV RNA after transfer to nitrocellulose and hybridization to a ^{32}P-labeled HDV-specific RNA probe. After hybridization, filters were washed, dried, and autoradiographed at −70°C for 12 h (HBV-HDV, S-HDV, and SM-HDV) or 48 h (SML-HDV) with an intensifying screen. The size of HDV genomic RNA (in kilobases) is indicated.

(Fig. 4). HDV RNA-containing particles derived from the Huh7SM cells were immunoprecipitated with anti-S as well as anti-pre-S2 antibodies, which demonstrated the presence of the M protein at the surface of the particle. HDV RNA-containing particles derived from the Huh7SML cells were immunoprecipitated with anti-S, anti-pre-S2, and anti-pre-S1 antibodies, which demonstrated the presence of the L protein in their envelopes and likely the presence of the M protein, since the pre-S2 domain of the L protein is not accessible to antibodies on the HBV viral envelope. As a control, HDV RNA-containing particles, termed HBV-HDV particles, derived from Huh7 cells transfected with pFC80 and pSVLD3, were immunoprecipitated with anti-S, anti-pre-S2, and anti-pre-S1 antibodies.

We concluded that the S-, SM-, and SML-HDV particles were similar in their HDV RNA and HDAg protein content and buoyant densities but different in the protein composition of their envelopes.

Infectivity of HDV-like particles produced in vitro. To determine infectivity of the HDV-like particles, primary cultures of chimpanzee hepatocytes were exposed to the S-, SM-, and SML-HDV-like particles. As a positive control, we used HDV-HBV particles previously shown to be infectious in our in vitro assay (37). Every 3 days after exposure, hepatocytes were examined for the presence of intracellular antigenomic HDV RNA by Northern blot hybridization. In three independent experiments, infections were seen only in hepatocytes exposed to SML particles as evidenced by the appearance of intracellular antigenomic HDV RNA (Fig. 5A) or increasing amounts of intracellular genomic RNA (Fig. 5B). Particles coated with either the S or the SM envelope proteins were not infectious even when inocula of higher titer than the SML-HDV inocula were used (Fig. 5B). The amount of HDV RNA in each inoculum was measured by

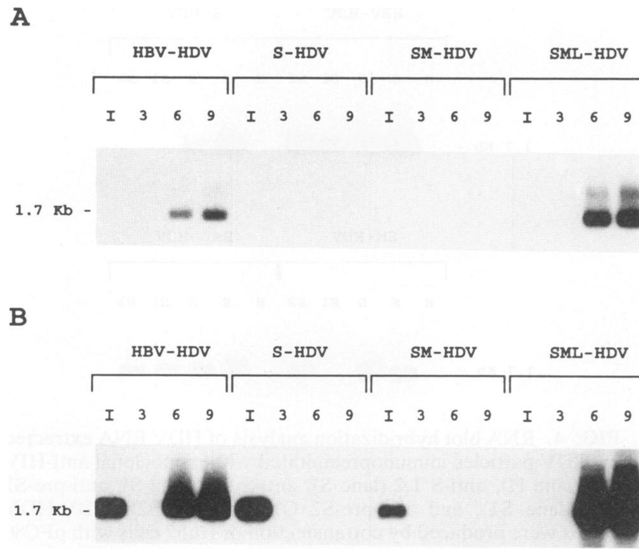

FIG. 5. RNA blot hybridization analysis of HDV RNA extracted from primary hepatocytes exposed to HBV-HDV, S-HDV, SM-HDV, or SML-HDV particles. In this experiment, 10^6 cells were exposed to 10^6, 10^7, 10^6, and 10^5 HBV-, S-, SM-, and SML-HDV particles, respectively. Total cellular RNA (5 μg) extracted from hepatocytes harvested 3, 6, and 9 days after exposure to the inocula (lanes 3, 6, and 9) was analyzed for the presence of antigenomic HDV RNA (A) or genomic HDV RNA (B). RNA extracted from the inocula was analyzed under the same conditions (lane I). RNA was separated on a 1.5% agarose–2.2 M formaldehyde gel and analyzed for the presence of genomic or antigenomic HDV RNA after transfer to nitrocellulose and hybridization to a ^{32}P-labeled HDV-specific RNA probe. After hybridization, filters were washed, dried, and autoradiographed at −70°C for 12 h with an intensifying screen. The size of HDV genomic and antigenomic RNA (in kilobases) is indicated.

RNA blot hybridization and scanning with a densitometer. Although HDV RNA present in the SML inoculum is not visible on the autoradiogram in Fig. 5, it was possible to detect it on a longer exposure (data not shown). In this particular experiment, it was estimated that the S-HDV inoculum contained 10^7 HDV genomes per ml, which is approximately 10 and 100 times greater than the titers of SM-HDV and SML-HDV inocula, respectively. Genomic HDV RNA could not be detected in hepatocytes harvested on day 9 after infection with the S and SM particles, whereas hepatocytes harvested on day 9 after infection with SML particles contained a high level of genomic RNA as evidence of infection. This RNA sample could be diluted 100 times before the signal disappeared (data not shown) by RNA blot hybridization assay. We therefore estimated that the SML-HDV-like particles were at least 1,000 times more infectious than the SM particles and at least 10,000 times more infectious than the S particles.

These results indicated clearly that the L protein is an important element of the envelope of infectious HDV particles.

DISCUSSION

Primary cultures of human or chimpanzee hepatocytes allow for the study of infections with HDV in a simple in vitro model system (36, 37). In the present study, we investigated the role of HBV viral envelope proteins in the assembly and infectivity of HDV.

We previously developed an assay for in vitro infection by measuring viral RNA present in the hepatocytes after exposure to an inoculum. The amount of intracellular HDV RNA was proportional to the amount of viral genomic RNA in the inoculum and to the duration of exposure to the inoculum (36).

Using Huh7 cells for production of HDV particles in the absence of HBV Dane particles, we showed here that an HDV inoculum need not contain Dane particles for initiation of infection. Since HBV does not provide a helper function in the replication of HDV RNA either (16), it is required only to provide the envelope proteins for particle assembly. We confirmed that HDV-like particles containing the HDV genome can be assembled in the presence of the S envelope protein only, and we demonstrated that the L HBV envelope protein must be included for the particle to be fully infectious. We have used S-HDV- and SM-HDV-like preparations of high titers for infection assays to demonstrate that these particles do not elicit infection in non-HBV-infected hepatocytes. We verified that all three types of particles contained genomic RNA and the two HDAg proteins p24 and p27 in the same proportions and that they all had a buoyant density similar to that of serum-derived virus. Since they differ from the SML particles only in the composition of their envelope, we concluded that this difference, the presence of the L protein, was solely responsible for the difference in infectivity.

Previous studies have described the topology of all three HBV envelope proteins and their role in the assembly of HBV particles (4, 5, 9, 10). The L protein is required to assemble Dane particles (4, 41), and the pre-S1 and the pre-S2 domains of the L and M proteins, respectively, are located at the surface of the HBV virion, making the pre-S domain a good candidate for containing a receptor-binding site (13). Since the envelopes of both mature HBV and HDV include the L protein, it will be interesting to determine whether the latter fulfills the same function in HBV as in HDV with regard to infection events.

The M protein has been ascribed a site for indirect binding through polymerized albumin (21, 29), but it remains unclear whether this property is indeed required for the formation and infectivity of Dane particles (4, 41). Clearly, the M protein is dispensable for assembly of HDV particles as confirmed here (34, 42). Whether it is required along with the L and S proteins for infectivity will be addressed in future studies by producing HDV-like particles with an LS envelope composition.

In general terms, for an enveloped virus such as HBV or HDV to infect a cell, the particle must bind to the cell membrane and then be internalized either by fusion with the cell membrane or by endocytosis (23). The L protein may mediate this event for both HBV and HDV infection in binding directly to the receptor as suggested for HBV or in promoting internalization by activation of a fusogenic site. Several reports have suggested that the pre-S1 domain of the L protein can indeed mediate binding of HBV to hepatocyte membranes. In other studies, chimpanzees were immunized with pre-S1 specific synthetic peptides, and antibodies against pre-S1 neutralized an HBV inoculum (26, 40). For these reasons, the pre-S1 domain has been proposed as the carrier of the HBV receptor-binding site.

Although our previous study on HDV infection in vitro has also shown that anti-pre-S1 antibodies neutralized HDV (37), the ability of anti-pre-S2 and anti-S antibodies (35, 37)

to preclude HDV infection with the same efficiency suggests that neutralization may be the result of particle agglutination, rather than antibodies binding to a receptor-binding site on the viral envelope. However, since the pre-S1 polypeptide is by definition unique to the L protein, it does represent a good candidate for bearing the receptor-binding site for HDV. Alternatively, the presence of pre-S1 in the envelope may activate a receptor-binding site located on either the pre-S2 or S domains of the L, M, or S protein. It will be interesting to investigate the binding capacity of each type of HDV particle (S, SM, SML) to hepatocyte membranes to determine whether the L protein's function in infectivity resides in initial binding or in a subsequent event. Better evidence for the presence of a pre-S1 binding site may result from deletions and mutations in this region and infection assays with the resulting HDV mutants. As demonstrated by the ability of monoclonal anti-pre-S1 and anti-pre-S2 antibodies to immunoprecipitate HDV RNA-containing particles, the pre-S1 and pre-S2 domains are exposed on the surfaces of HDV and Dane particles, indicating that the composition of the infectious HDV particle envelope and the topology of the L and M proteins are similar to those of infectious HBV. The difference in viral uptake between the two particles may reside in the uncoating process of a bound particle, rather than in receptor binding.

In the present study, we also confirmed that the S protein is sufficient for assembly of an HDV RNA-containing particle, suggesting that the production of infectious HDV may occur in patients who have cleared HDV DNA replication but still express the L, M, and S proteins from integrated viral DNA sequences. Furthermore, it is likely that an HDV-positive serum contains various types of particles which may differ by their content of HDAg proteins and HDV RNA (34) and by the protein composition of their envelope (S, SM, SML, or SL). As a result, the infectivity of a serum may not be proportional solely to its HDV RNA titer but also to the proportion of L protein. Since some HBV subtypes have been reported to produce more of the L protein than others (4), this would translate to a degree of HDV infectivity that differs according to the subtype of helper HBV.

Our data demonstrate for the first time that the HBV L protein is necessary for infectivity of a viral particle coated with HBV envelope proteins. However, because of the lack of evidence for in vitro infection of chimpanzee hepatocytes with HBV, the role of the L protein in infectivity of Dane particles could not be addressed. Studies of the duck hepatitis virus, another member of the HBV family, have demonstrated that the L duck hepatitis virus envelope protein is important for infectivity and that myristylation of the pre-S1 domain at its amino terminus is essential in this process (22).

Assuming that the L protein's function resides in binding to the receptor, our results indicate that empty 22-nm-diameter HBV subparticles present in large amounts in HDV infectious serum would be unable to compete with HDV for cell membrane receptors because of their lack of the L protein. This assumption will be tested by binding assays with HDV particles produced in vitro and chimpanzee hepatocyte membranes. Such binding assays have been performed with hepatocyte membranes or with the human hepatoma cell line HepG2 and have so far indicated a specific affinity for the pre-S1 polypeptide (8, 20, 25, 28–30).

It will be interesting to determine, with more sensitive assays, whether HDV-like particles coated with the S or SM proteins have the ability to infect hepatocytes at low efficiency, and whether the woodchuck HDV pseudotype (24, 31), which is coated with woodchuck hepatitis virus envelope proteins, is also capable of infecting chimpanzee hepatocytes. The answers to these questions may aid in understanding why woodchucks chronically infected with woodchuck hepatitis virus are susceptible to infection with a human HDV, but resistant to infection with HBV. One likely explanation is that only one or a few HDV particles need enter a woodchuck hepatitis virus-infected liver cell, by a nonspecific mechanism such as endocytosis, to produce progeny HDV pseudotypes coated with woodchuck hepatitis virus envelope proteins. Such particles would, in turn, be fully infectious and able to spread infection through specific virus-receptor interaction. The S or SM HDV particle may well elicit HDV infection by the same mechanism when exposed to HBV-infected hepatocytes.

Our model represents the most appropriate experimental system in which to assay infectivity of HDV particles since it does not permit amplification of the HDV RNA signal by spread of the infection. When, as in this study, hepatocytes are derived from a non-HBV-infected animal and the inocula do not contain Dane particles, viral infection leads to HDV RNA replication without production of progeny virus and cell-to-cell spread of the infection. The resulting level of intracellular viral RNA is thus the product of a single cycle of infection and is therefore proportional to the ability of a given HDV particle to enter the hepatocyte. Using this experimental approach, we demonstrated that the presence of the L protein in the envelope of HDV particles increases infectivity at least 1,000 to 10,000 times, presumably by allowing for a specific virus-hepatocyte interaction. The tissue culture model described here will be utilized to further define the interaction between HDV and hepatocyte membranes and to narrow the domain of the pre-S1 region responsible for infectivity.

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