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The Middle Hepatitis B Virus Envelope Protein Is Not Necessary for Infectivity of Hepatitis Delta Virus

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The hepatitis delta virus (HDV) envelope contains the large (L), middle (M), and small (S) surface proteins encoded by coinfecting hepatitis B virus. Although HDV-like particles can be assembled with only the S protein in the envelope, the L protein is essential for infectivity in vitro (C. Sureau, B. Guerra, and R. Lanford, J. Virol. 67:366–372, 1993). Here, we demonstrate that the M protein, previously described as carrying a site for binding to polymerized human albumin, is not necessary for infectivity. HDV-like particles coated with the S plus L or the S plus M plus L proteins are infectious in primary cultures of chimpanzee hepatocytes. We conclude that the S and L proteins serve two essential functions in the HDV replication cycle; the S protein ensures the export of the HDV genome from an infected cell by forming a particle, and the L protein ensures its import into a human hepatocyte.

The 36-nm-diameter hepatitis delta virus (HDV) particle is coated with an outer envelope of hepatitis B virus (HBV) origin and contains an inner ribonucleoprotein complex made of a circular single-stranded RNA genome and two proteins with molecular masses of 24 and 27 kDa, respectively, bearing the hepatitis delta antigen (HDAg) (1, 5, 19, 22). The HDV genome lacks the coding capacity for its own envelope proteins but codes for the HDAg proteins p24 and p27, the only HDV-encoded proteins known to date (22, 23).

The envelope of HDV is composed of lipids and three related viral proteins that are designated large (L), middle (M), and small (S), which are translated from a single open reading frame on the HBV genome (6). From amino to carboxy terminus, the L protein contains the pre-S1 (119 amino acid residues), pre-S2 (55 amino acid residues), and S (226 amino acid residues) domains. The M protein contains both the pre-S2 and the S regions, whereas the S protein contains only the S region. As in HBV particles, the pre-S1 and pre-S2 domains 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 (17). Therefore, the M and L proteins may be involved in binding to the viral receptor on the cell membrane. Recent studies have demonstrated that the S and L proteins, but not the M protein, were required to assemble mature HBV particles (2, 3). In contrast, the S protein alone was sufficient to provide an envelope for the packaging of HDV RNA and HDAg proteins to form an HDV-like particle (13, 15, 21, 24). However, the presence of the L protein in the envelope of such particles was shown to be essential for infectivity (15). Particles coated with the S, M, and L envelope proteins (SML particles) were infectious in primary cultures of chimpanzee hepatocytes, whereas particles coated with S and M proteins (SM particles) or coated with the S protein only (S particles) were not.

In the present study, we addressed the role of the M protein in the infectivity of HDV particles. At its amino terminus, this protein contains the pre-S2 polypeptide (pre-S2–M), which is capable of binding to polymerized albumin. Such binding has been postulated to mediate indirect attachment to the cell membrane, thereby providing a means of entry into the hepatocyte (6). The pre-S2 polypeptide is also present in the L protein (pre-S2–L) and is located between the pre-S1 and the S domains. Although it is believed not to be exposed on the outside of the particle (6), it may have a binding function when exposed by an activation mechanism. Recent studies indicate that the M protein is not required to assemble HBV particles that are infectious in primary cultures of human hepatocytes (3). These results would preclude any essential function of pre-S2–M in the morphogenesis or infectivity of HBV. In addition, HBV mutants with substantial deletions in the pre-S2 domain have been recovered from human sera (4, 14, 20). Some of these deletions are within the albumin-binding site. However, HBV particles coated with such envelope proteins have not been tested for infectivity.

The present analysis was performed in an in vitro model system in which HDV-like particles coated with an envelope containing the S, M, and L proteins and particles coated with an envelope containing the S and L proteins were produced in Huh7 cells by transfection with HBV DNA and HDV cDNA. They were then assayed for infectivity in primary cultures of chimpanzee hepatocytes. The results indicate that both types of particles are equally infectious, therefore demonstrating that the M envelope protein is dispensable for assembly and infectivity of HDV virions.

For production of HBV envelope proteins in Huh7 cells, two expression vectors were constructed. A *Bgl*II (nucleotide 2840)-to-*Bgl*II (nucleotide 1987) fragment of HBV DNA (*ayw* subtype) was inserted between the *Bam*HI site of the pT7E19(+) plasmid (12). The resulting recombinant plasmid, pT7HB2.7, contains the pre-S1–pre-S2–S gene and can direct the expression of the S, M, and L proteins. It includes the HBV promoter upstream of the pre-S1 region for expression of the mRNA for the L protein, the HBV promoter within the pre-S1 region for expression of the M and S mRNAs, and the HBV polyadenylation signals. Plasmid pT7HB2.7M(−) is derived from plasmid pT7HB2.7; however, the pre-S2 ATG has been mutated to ACG, thereby preventing the expression of the M protein. In vitro mutagenesis was performed by the PCR technique with two complementary mutagenic oligonucleotides according to the overlap extension method that has been described by Higuchi et al. (7). PCR-generated fragments were

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cloned in pT7HB2.7 and sequenced. A clone, designated pT7HB2.7M(−) and containing the mutation at the pre-S2 ATG and no additional mutations within the PCR-generated fragment, was selected and used for subsequent transfections.

For production of HDAg and HDV RNA, we used the recombinant plasmid pSVLD3 which 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 (9, 18).

For production of HDV particles, Huh7 cells were transfected with a mixture of cloned HDV cDNA (pSVLD3) and HBV DNA [pT7HB2.7 or pT7HB2.7M(−)] by using lipofectin according to the manufacturer's directions (GIBCO BRL, Life Technologies, Inc.). The cells were propagated in a 1/1 mixture of Dulbecco's modified Eagle medium and F12 medium supplemented with 10% fetal bovine serum. They were seeded at 10^6/35-mm-diameter well and were transfected at 24 h postseeding. The cells were exposed to the DNA-lipofectin mixture for 2 h and were washed with phosphate-buffered saline (PBS) before incubation in fresh medium. Culture medium was harvested on days 3, 6, and 9 after transfection and was analyzed for the presence of HDV RNA by hybridization to an HDV-specific probe. Transfections were carried out with either 10 μg of HDV recombinant plasmid pSVLD3 and 10 μg of pT7HB2.7 for production of particles coated with the S, M, and L envelope proteins or with 10 μg of pSVLD3 and 10 μg of pT7HB2.7M(−) for production of particles coated with the S and L proteins.

Particles produced in the culture medium of Huh7 cells transfected with pSVLD3 plus pT7HB2.7 or pSVLD3 plus pT7HB2.7M(−) were subjected to protein analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then to immunoblotting with an anti-hepatitis B surface antigen (HBSAg) antibody. Sedimented particles were disrupted in 100 μl of buffer containing 2% SDS and 2% β-mercaptoethanol and were heated at 100°C for 5 min. Immunoblotting analysis was performed as described elsewhere (10) with rabbit anti-HBsAg antibodies (Calbiochem, San Diego, Calif.). The S proteins appeared as 24- and 27-kDa polypeptides, the M proteins were detected to have molecular masses of 35 to 36 kDa, and the L proteins were detected to have molecular masses of 39 and 42 kDa. As expected, only the S and L proteins were detected in SL-HDV particles released in the culture medium of cells transfected with pT7HB2.7M(−), whereas the S, M, and L proteins were present in particles (SML-HDV) recovered after transfection with pT7HB2.7.

Sedimented particles were also analyzed for the presence of HDAg polypeptides using a human anti-HDAg antibody. HDAg proteins were detected (Fig. 1) in both SL and SML particles at a similar ratio of p24 to p27, as estimated by scanning the autoradiogram with a laser densitometer.

For further characterization of HDV-like particles produced by Huh7 cells, the culture medium was first clarified by centrifugation at 5,000 × *g* for 1 h at 4°C. 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. An aliquot of each fraction was used for detection of HBsAg by an enzyme-linked immunosorbent assay and HDV RNA by Northern (RNA) blot analysis (Fig. 2).

HBsAg and HDV genomic RNAs were detected at densities of 1.24 and 1.20 g/cm^3 (fractions 5 and 6) for both SL-HDV and SML-HDV particles. These results indicated that HDV RNA with genomic size and polarity was detected at a density characteristic of serum-derived HDV particles.

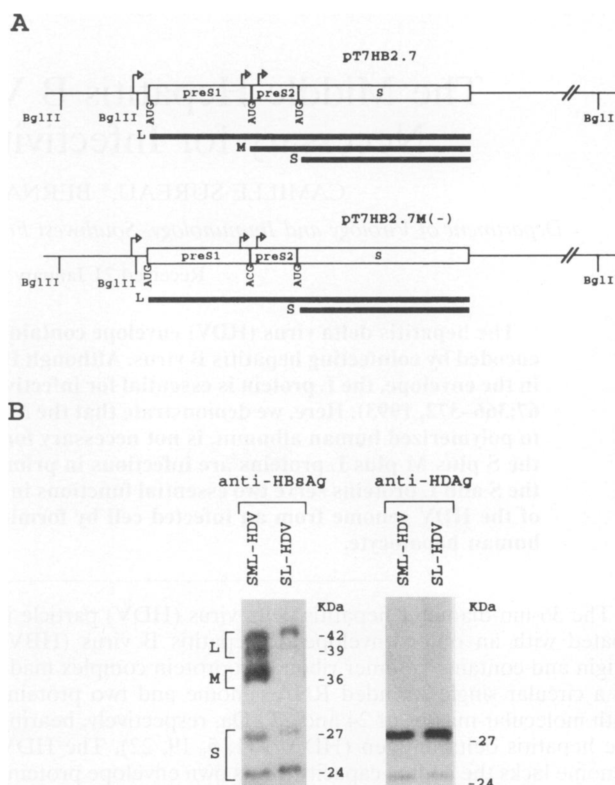

FIG. 1. (A) Recombinant plasmids pT7HB2.7 and pT7HB2.7M(−) direct the synthesis of the S plus M plus L and S plus L HBV envelope proteins, respectively. Horizontal dark bars, the S, M, and L proteins; plain lines, HBV sequences; dashed lines, plasmid sequences; open boxes, HBV envelope open reading frame divided in pre-S1, pre-S2, and S domains. AUG start codons for the L, M, and S genes are indicated. Initiation of transcription is designated by arrows. (B) Immunoblot analysis of HBsAg (left panel)- and HDAg (right panel)-related proteins extracted from culture medium of Huh7 cells transfected with pT7HB2.7 and pSVLD3 (SML-HDV) or transfected with pSVLD3 and pT7HB2.7M(−) (SL-HDV). Particles were sedimented from 30 ml 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 a polyvinylidene difluoride membrane (Flurotrans; Pall Biosupport Division, Glen Core, N.Y.), and analyzed as described elsewhere (10) using either rabbit anti-HBsAg or human anti-HDAg antibodies. The molecular masses of the S, M, and L 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).

We concluded that the SL-HDV and SML-HDV particles were similar in their HDV RNA and HDAg protein contents and buoyant densities and differed only by the presence or absence of the M protein in their envelopes.

To determine the infectivity of SL and SML particles, hepatocytes isolated from a wedge biopsy obtained from a chimpanzee were used as susceptible cells. The animal was 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

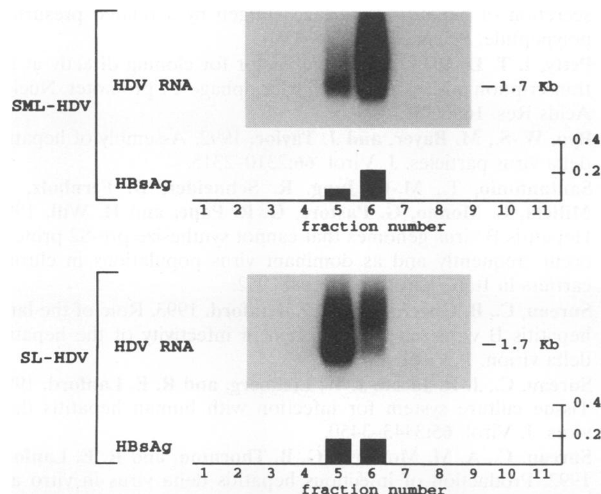

FIG. 2. Analysis of particles derived from culture medium of Huh7 cells transfected with pT7HB2.7 and pSVLD3 (SML-HDV) or transfected with pSVLD3 and pT7HB2.7M(−) (SL-HDV). Particles were sedimented from 30 ml of culture medium as described in Materials and Methods. The pellet was resuspended in PBS, 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, one-third of each fraction was used for RNA extraction, and one-third was used for detection of HBsAg by enzyme-linked immunoassay (Auzyme EIA; Abbott Laboratories, North Chicago, Ill.). RNAs were isolated, 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-labelled HDV-specific RNA probe. Following hybridization, the filters were washed, dried, and autoradiographed at −70°C for 12 h with an intensifying screen. The numbering (1 to 11) of each fraction is indicated. Fractions 5 and 6 correspond to densities of 1.24 and 1.20 g/cm^3, respectively. The sizes of HDV genomic RNAs (in kilobases) are indicated. The amounts of HBsAg are expressed as A_{492}.

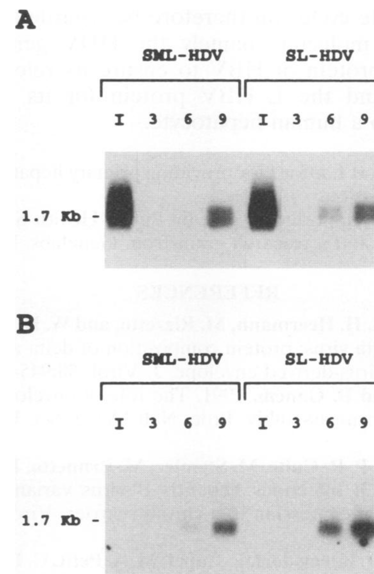

FIG. 3. RNA blot hybridization analysis of HDV RNAs extracted from primary hepatocytes exposed to SML-HDV or SL-HDV particles. In this experiment, 10^6 cells were exposed to 10^7 genomes of SML-HDV and SL-HDV particles, respectively. Total cellular RNAs (5 μg) extracted from hepatocytes that were harvested 3, 6, and 9 days after exposure to the inocula (lanes 3, 6, and 9) were analyzed for the presence of genomic HDV RNA (A) or antigenomic HDV RNA (B). RNAs extracted from the inocula were analyzed under the same conditions (lane I). RNAs were 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-labelled HDV-specific RNA probe. Following hybridization, the filters were washed, dried, and autoradiographed at −70°C for 12 h with an intensifying screen. The sizes of HDV genomic and antigenomic RNAs (in kilobases) are indicated.

been described previously (8, 16). The cells were exposed to HDV for at least 12 h on day 3 postseeding. For infection, culture medium collected from Huh7 cultures at 3, 6, and 9 days posttransfection was pooled and concentrated in a Centricon 100 microconcentrator. The concentrate was resuspended in 1 ml of serum-free medium, resulting in a 5× concentration, and was then added to a 22-mm-diameter well containing 10^6 hepatocytes. By measurement of HDV RNA extracted from the inoculum and by comparison with a known amount of HDV cDNA, we estimated that both inocula contained approximately 10^7 HDV genomes per ml. Following exposure to the inoculum, the cells were washed and incubated in 1 ml of fresh serum-free medium. The cells were harvested every 3 days thereafter for detection of intracellular HDV RNA by RNA blot hybridization as described previously (18). Infection was evidenced by the appearance of increasing amounts of intracellular genomic RNA (Fig. 3A) and antigenomic RNA (Fig. 3B) in hepatocytes that were exposed to SL or SML particles. Thus, both SL-HDV and SML-HDV particles appeared to be infectious in primary hepatocytes, which indicates clearly that the M protein is not required in the envelope of infectious virion. However, it remains possible that particles containing the M protein have a higher specific infectivity than those lacking it and that the presence of serum in the inoculum interferes with infectivity. This can be determined by comparing SL and SML particles in the linear range of infectivity assay and by adding human serum to the inocula.

We previously demonstrated that HDV-like particles coated with the S or S plus M proteins were significantly less infectious (if at all) than particles coated with all three S, M, and L proteins. These results demonstrated that the L protein is essential for infectivity. In contrast, we have shown here that the M protein has no essential role in infectivity when tested in vitro in the absence of serum. These results were confirmed by using different preparations of SL and SML particles as well as primary hepatocytes isolated from different animals.

The pre-S2 polypeptide has been shown to contain a site for binding to polymerized albumin, suggesting that it could allow for indirect binding of the particle to the hepatocyte membrane. Our findings clearly demonstrate that pre-S2–M has no essential function in either binding of the particle to the hepatocyte or subsequent entry events. However, pre-S2–L may play such a role in the infection process. This question will be explored by testing HBV mutants coated with SL envelope from which pre-S2–L has been deleted.

Whatever the precise function of the pre-S2 polypeptide, we are now able to better understand the role of each HBV envelope protein in the life cycle of HDV: (i) the S protein is necessary to assemble an HDV RNA-containing particle (13, 15, 24) but is not sufficient to confer infectivity (15); (ii) the L protein, while unable alone to secrete a particle (11), must be included in the envelope of HDV for infectivity; (iii) the M protein is clearly not essential at any stage of the HDV replication cycle.

The HDV life cycle can therefore be regarded as that of a circular RNA molecule, namely the HDV genome, which utilizes the S protein of HBV to ensure its release from an infected cell and the L HBV protein for its introduction specifically into a human hepatocyte.

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