Phenotypic Mixing of Rodent but Not Avian Hepadnavirus Surface Proteins into Human Hepatitis B Virus Particles

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The virus family *Hepadnaviridae* comprises two genera: orthohepadnaviruses isolated from humans (hepatitis B virus [HBV]) and rodents (e.g., woodchuck hepatitis virus [WHV]) and avihepadnaviruses isolated from birds (e.g., duck hepatitis B virus [DHBV]). They carry in their envelopes two (DHBV) or three (HBV and WHV) coterminal proteins referred to as small (S), middle (M), or large (L) surface protein. These proteins are also secreted from infected cells as subviral particles consisting of surface protein and lipid (e.g., 20-nm hepatitis B surface antigen for HBV). To investigate the assembly of these proteins, we asked whether surface proteins from different hepadnaviruses are able to mix phenotypically with each other. By coexpression and coimmunoprecipitation with species-specific antibodies, we could show the formation of mixed subviral particles and disulfide-linked heterodimers between the WHV S and HBV M proteins whereas the DHBV and HBV surface proteins did not coassemble. Complementation of HBV genomes defective in expressing the S or L protein and therefore incompetent to form virions was possible with the closely related WHV S protein or a WHV pre-S–HBV S chimera, respectively, but not with the less related DHBV S or L protein or with a DHBV L–HBV S chimera. The results suggest that the assembly of HBV subviral particles and virion envelopes requires relatively precise molecular interactions of their surface proteins, which are not conserved between the two hepadnavirus genera. This contrasts with the ability of, e.g., rhabdoviruses or retroviruses, to incorporate envelope proteins even from unrelated viruses.

During a double infection of a single host cell with two different viruses, a phenomenon called phenotypic mixing may be observed: newly produced virus particles may contain the genome of one virus and structural proteins encoded by both viruses (32). It is generally accepted that the assembly of a complex macromolecular structure like a virion is a highly ordered process driven by specific interactions of the protein subunits. Therefore, the phenotypic mixing of the two viruses would be expected to reflect the relationships of their structural proteins. Surprisingly and seemingly in conflict with this view, envelope proteins of viruses belonging to very different families, like retroviruses, paramyxoviruses, and togaviruses, can be incorporated into the envelope of, e.g., rhabdoviruses or retroviruses, whereas most cellular proteins are excluded (for a review, see reference 33). These observations led to a model which proposed that the mechanism of interactions among viral envelope proteins and between these proteins and internal structures of virions is conserved within virtually all virus families (33). However, for some viruses, like the alphavirus Sindbis virus, incorporation of foreign envelope proteins into the virion could not be detected (34) and efficient mixing of alphavirus spike complexes into the envelope of a type C retrovirus was observed only when the complex was expressed at high densities at the cell surface (29).

The human hepatitis B virus (HBV) is the prototype of a family of enveloped viruses named *Hepadnaviridae* defined only several years ago (14, 16). Other members of this family have been isolated from rodents (woodchuck hepatitis virus [WHV] and ground squirrel hepatitis B virus) and birds (duck hepatitis B virus [DHBV] and heron hepatitis B virus). Hep-

adnaviruses cause acute or chronic infections of the liver and are relatively species specific; e.g., HBV can be transmitted experimentally just to higher primates like chimpanzees (22). The structure and composition of the virions are quite similar: icosahedral nucleocapsids formed by multiple copies of a single capsid protein (6) contain a circular partially double-stranded 3.0- to 3.3-kb DNA genome and a virus-encoded DNA polymerase. The genome of hepadnaviruses is similarly organized and is generated by a unique mechanism which includes reverse transcription of a terminal-redundant, pre-genomic RNA molecule (for a review, see reference 12). The nucleocapsids are surrounded by an envelope containing presumably lipid and two or three surface proteins. The virus family is divided into the genera *Orthohepadnavirus* and *Avihepadnavirus* comprising the mammalian and avian viruses, respectively. This division is based on the nucleotide and amino acid sequence homology, the presence of an additional gene X of unknown function in orthohepadnaviruses, and the fact that orthohepadnaviruses carry three surface proteins and avihepadnaviruses carry two surface proteins.

These surface proteins are expressed without splicing from a single open reading frame by means of different in-frame start codons (Fig. 1A). Therefore, the amino acid sequence of the smallest surface protein, called S, is repeated at the C terminus of the largest one, referred to as L, which carries an additional N-terminal sequence named pre-S. The mammalian hepadnaviruses carry a third start codon located within the pre-S sequence which is used to translate a middle-sized surface protein referred to as M. This start codon divides the pre-S region into an N-terminal pre-S1 and a C-terminal pre-S2 domain (15). To distinguish the different surface proteins of the species, the single letters S, M, and L are followed by HBs for human hepatitis B surface, WHs for woodchuck hepatitis surface, or DHBs for duck hepatitis B surface protein; e.g., MHBs refers to the middle surface protein of HBV. The DHBs pro-

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FIG. 1. Domains and homologies of the hepadnaviral surface proteins and authentic as well as fused animal virus surface proteins used in this work. Sequences of the HBV, WHV, and DHBV surface proteins are shown as solid, open, and hatched boxes, respectively. (A) The three HBV surface proteins SHBs, MHBs, and LHBs are expressed from a single open reading frame by three in-frame start codons. Therefore, the 24-kDa SHBs protein consists of the S domain (thin box), the 30-kDa MHBs protein carries an additional N-terminal pre-S2 sequence (thicker box), and the 39-kDa LHBs protein carries yet an additional N-terminal sequence, referred to as pre-S1 (thickest box). The WHV surface proteins SWHs, MWHs, and LWHs are organized in the same way. DHBV has only one pre-S domain and consequently only two surface proteins. SDHBs and LDHBs. The black lines below the boxes indicate the positions of the N-terminal type I and central type II translocation signals in the S domain which determine the transmembrane folding of the surface proteins (3, 7, 8, 24). The vertical lines connect homologous regions of the surface proteins. The numbers with percentage signs show the proportions of identical amino acids at homologous positions within the regions limited by the left and right vertical lines. n.d., not detectable. (B) In this work, the SWHs protein, a fusion construct consisting of the WHV pre-S1-pre-S2 and HBV S domain (WH), the SDHBs, the LDHBs, and a fusion construct consisting of the DHBV pre-S and the HBV S domain were tested for their ability to be incorporated into subviral 20-nm HBsAg particles (Fig. 2 to 7) and into the virion envelope (Fig. 8).

teins are not N glycosylated, the S domains of the mammalian viruses are partially N glycosylated, and the pre-S2 domain is N glycosylated in MHBs but not in LHBs (15). The S domains contain two hydrophobic stretches (Fig. 1A) which for HBV have been shown to be part of a type I and type II translocation signal, respectively (7, 8). After synthesis at the membrane of the endoplasmic reticulum (ER), the SHBs and MHBs proteins traverse the membrane twice with a cytosolic loop between the two translocation signals. In the LHBs protein, however, the pre-S1, pre-S2, and probably also the N-terminal region of S up to signal II are first located at the cytosolic side of the ER (3, 24). The hepadnavirus envelope proteins show different levels of homology in their domains (Fig. 1A): the most conserved region is the one between the two translocation signals in the S domain, with 66 and 38% amino acid identities for HBV and WHV and HBV and DHBV, respec-

tively. Less conserved are the pre-S1-pre-S2 domains of HBV and WHV, and the DHBV pre-S domain has no primary sequence homology at all with the HBV pre-S region.

One special feature of hepadnaviruses is that their surface proteins are not only incorporated into the outer shell of the virions but, in addition, form subviral lipoprotein particles with a spherical or tubular shape which are released from infected cells without cell lysis in great excess over virions. These particles derive from transmembrane surface proteins (28), assemble at membranes of a pre-Golgi compartment (17), and are released from the cells by the constitutive secretory pathway. They do not contain detectable amounts of host proteins (15). The morphogenesis of the virion has been studied only recently. The current model assumes that the icosahedral nucleocapsid preassembles and matures in the cytosol and interacts subsequently with transmembrane surface proteins. After budding, the luminal virion is supposed to be exported by the same pathway as that for subviral particles (11).

In this article, we show that surface proteins of the closely related mammalian WHV but not of the less related avian DHBV combine with human HBs proteins to form mixed subviral particles and virions. This suggests that the assembly process of hepadnavirus particles requires very precise interactions of the protein subunits which are not conserved within this virus family. The morphogenesis of hepadnavirus envelopes is therefore different from the less stringent assembly of, e.g., rhabdovirus or retrovirus envelopes.

MATERIALS AND METHODS

Plasmid constructions. The numbering of the HBV genome used for this study (30) starts with the unique *EcoRI* site. Plasmid pSVM carries the fragment of the HBV genome containing the pre-S2 region and the S gene (*Bsu36I* [nucleotide (nt) 3201] to *BglII* [nt 1982]) inserted into the *HindIII-BamHI* sites of the simian virus 40 (SV40) early promoter expression vector pSV65 (25). The S start codon in this plasmid was changed from ATG (methionine) to ACG (threonine), causing the expression of the MHBs protein derivative only, without concomitant SHBs synthesis. Plasmid pSV45H contains the pre-S1-pre-S2-S open reading frame of the HBV genome cloned into the expression vector pSV65 and directs the expression of LHBs, MHBs, and SHBs proteins (25). Plasmids pRVS⁺ and pRVL⁻ contain one and a half copies of the HBV genome in a head-to-tail configuration, with an ATG-to-ACG mutation of the S gene initiation codon and a stop codon in the 52nd codon of the pre-S1 frame, respectively (2). The cloned genome of WHV (10) was kindly provided by M. Roggendorf as plasmid WHV I. The numbering of the WHV sequence starts with the unique *EcoRI* site. Plasmid pSV24W contains the 1,973-bp *TaqI-SacI* (nt 263 to 2236) fragment of WHV I inserted into the *SacI-AccI* sites of vector pSV65 and drives the synthesis of the SWHs protein. Plasmid pSVWH carries an exact fusion of the WHV pre-S1 and pre-S2 region with the HBV S gene cloned into pSV65. The fusion was generated by first PCR amplifying an SV40-WHV pre-S fragment from plasmid WHV1/13 containing a *SacI* (nt 2236)-linearized monomer of the WHV genome cloned into the *SacI* site of pSV65. The insert was oriented so that the SV40 transcript had sense polarity relative to the HBV genome. The PCR fragment was synthesized with the primer 5'-AGCTATTCCAGAAGTAGTGA (primer 1) annealing to the SV40 sequence and downstream primer 5'-GATGTTCCTCATCTCCGGTGACTGTGCAGGG annealing with its 3' part to the 3' end of WHV pre-S2. Second, a fragment from HBV DNA was PCR generated with upstream primer 5'-TCACCGAGATGGAGAACATCAGATCAGGAT and downstream primer 5'-GAAAGCCTGTGTTAGC (primer 2) annealing to the HBV sequence from nt 1076 to 1090. The two PCR fragments were spliced, which is possible because of their homologous terminal regions, by another PCR reaction with primers 1 and 2. The 1,575-bp PCR fragment was digested with *XbaI* in the HBV sequence and *BglII* at nt 2534 in the WHV sequence and cloned into pSVBX24 cut with *BamHI-XbaI*. Plasmid pSVBX24H contains the *PstI-BglII* (nt 25 to 1982) fragment of the HBV genome inserted into *PstI-BamHI*-digested pSV65 and directs the expression of SHBs. The *BamHI* site in this plasmid at nt 1396 downstream of the S gene has been destroyed by partial *BamHI* digestion, a filling up of the single-stranded ends with DNA polymerase, and religation so that the *BamHI* site at nt 26 is unique in this construct. The numbering of the DHBV genome (20) starts with the unique *EcoRI* site. Plasmid pSVS14 (kindly provided by K. Kuroki) contains the DHBV S gene on the *PvuI-DraI* (nt 1243 to 2959) fragment of the DHBV genome inserted into the *HincII* site of the expression vector pSV65 and directs the expression of SDHBs. Plasmid pSVPS4 (kindly provided by K. Kuroki) carries the *DraI-DraI* (nt 740 to 2959) fragment containing the pre-S and S genes of the DHBV genome

inserted into the *HincII* site of pSV65 and directs the expression of LDHBs. Plasmid pSVLH was constructed in a manner analogous to that of pSVWH by first PCR amplifying an SV40-DHBV pre-S fragment with primer 1 and primer 5'GATGTTCTCCATTTCTTCTTAGGGGAGG from plasmid pSVPSS4 and an HBV S gene fragment with primer 5'AAGAAGAAAATGGAGAAC ATCACATCAGGAT and primer 5'GTGAGTGATTGG (primer 3) annealing to the HBV sequence from nt 314 to 325 from plasmid pSVBX24H. The two fragments were spliced by PCR with primers 1 and 3, and the product was cut with *HindIII* and *XbaI* and inserted into *HindIII*-*XbaI*-digested pSVBX24H.

Expression and detection of hepadnavirus surface proteins in COS7 cell cultures. Transient transfections of COS7 cells, pulse-chase labeling with [³⁵S] methionine, immunoprecipitation with antibodies prebound to protein A-Sepharose, endoglycosidase treatment, gel electrophoresis, and autoradiography were done as described previously (4). For immunoprecipitation of hepatitis B surface antigen (HBsAg), a goat anti-HBs was used (DAKO). For the binding of SDHBs and LDHBs, an antiserum from a rabbit immunized with DHBsAg which was isolated from infected ducks was employed (kindly provided by S. Lottmann and W. H. Gerlich).

Two-dimensional gel electrophoresis. The immunoprecipitated sample was divided into two halves. One half was denatured with sodium dodecyl sulfate (SDS) and heat without the addition of a reducing agent and loaded on a 12-cm-long SDS-14% polyacrylamide tube gel (diameter, 3 mm). After electrophoresis, the gel was removed from the tube, covered with 0.3 ml of 0.5 M dithiothreitol, and incubated for 15 min in a sealed chamber. The gel tube was then fixed with 1% agarose dissolved in stacking gel buffer on top of an SDS-14% polyacrylamide slab gel. For reference, the second half of the sample and a molecular mass standard were heat denatured with SDS in the presence of dithiothreitol and loaded into pockets on the same gel.

Expression and detection of virions in HepG2 cell cultures. Transient transfections of HepG2 cells, immunoprecipitation of virus particles from the medium with an antibody against the nucleocapsid antigen (rabbit anti-hepatitis B core [HBc]; DAKO) in the absence or presence of 0.5% Nonidet P-40 and with anti-HBs as well as from the cell lysate with anti-HBc, labeling of the viral genome enclosed in the precipitated particles by the endogenous polymerase of the virus with [³²P]dCTP, isolation of the genome, and detection of the labeled genome by agarose gel electrophoresis and autoradiography have been described elsewhere (2).

RESULTS

Incorporation of the SWHs proteins into subviral HBV particles. Cell lines which are efficiently infectible by hepadnaviruses are not available, so the detection of pseudotypes by host range determination was not possible. Furthermore, cell lines transfected with cloned HBV DNA produce only low amounts of virions and are therefore not suitable for directly studying the protein composition of virions. Therefore, we decided first to investigate whether foreign proteins can be incorporated into subviral HBV particles. We started with the SWHs protein, which shows the greatest sequence homology to SHBs (Fig. 1A). Because SWHs and SHBs have nearly identical sizes and cannot be discriminated on polyacrylamide gels, we coexpressed the SWHs protein together with the larger MHBs protein in transiently transfected COS7 cells. We looked for the appearance of mixed subviral particles by metabolic labeling with [³⁵S]methionine and coimmunoprecipitation of SWHs with MHBs-specific antibodies (Fig. 2). Plasmid pSVM driving the expression of MHBs carried a missense mutation of the start codon for the SHBs protein to prevent concomitant expression of SHBs (Fig. 2, lanes 1 and 2). After 1 h of labeling and 24 h of chase, the unglycosylated, once-glycosylated, and twice-glycosylated forms of MHBs could be detected in cell lysates as 30-, 33-, and 36-kDa bands, respectively (Fig. 2, lane 1). Substantial amounts of the glycosylated forms of MHBs were secreted into the medium (Fig. 2, lane 2). The double-glycosylated form shifted in molecular mass, probably because of glycan modification. It is not known why the unglycosylated protein is retained within the cells. Both the MHBs proteins and the unglycosylated and glycosylated SWHs proteins with molecular masses of 24 and 27 kDa, respectively, could be detected in cell lysates (Fig. 2, lane 5) and medium (Fig. 2, lane 6) after coexpression. Since the polyclonal anti-HBs antibody used for immunoprecipitation did not cross-react with SWHs

FIG. 2. Mixed assembly of MHBs and SWHs to form subviral particles. COS7 cells were transfected with expression vectors for MHBs (lanes 1 and 2) and SWHs (lanes 3 and 4) and with both plasmids (lanes 5 and 6) and were mock transfected (lanes 9 and 10). After pulse-chase labeling with radioactive methionine, proteins were immunoprecipitated from cell lysates (L) and culture medium (M) with anti-HBs and depicted by polyacrylamide gel electrophoresis and autoradiography. The SWHs protein was coprecipitated by anti-HBs in the coexpression experiment (lanes 5 and 6), although this antibody did not cross-react with SWHs (lanes 3 and 4). The coprecipitation is not due to unspecific aggregation of otherwise separate particles because immunoprecipitation of material mixed after expression in different cultures yielded only MHBs protein (lanes 7 and 8).

(Fig. 2, lanes 3 and 4), we infer that coexpression led to the formation of mixed complexes in the cell lysate and mixed particles in the medium. The coprecipitation was not caused by unspecific association of otherwise separated MHBs and SWHs particles because immunoprecipitation of a mixture of cell lysates or medium from separately transfected cultures yielded only the MHBs protein (Fig. 2, lanes 7 and 8).

MHBs and SWHs form disulfide-linked heterodimers. During the biosynthesis of subviral particles, the SHBs protein forms disulfide-linked homodimers in the ER membrane (17) and heterodimers with MHBs and LHBs (31). We asked whether MHBs and SWHs were also able to form mixed dimers during coexpression. This was anticipated because the proteins form mixed particles (Fig. 2) and most of the cysteine residues in SHBs and SWHs are conserved. For the analysis, the MHBs and SWHs proteins were radioactively labeled with methionine for 10 min without chase, immunoprecipitated with polyclonal anti-HBs from a Nonidet P-40 cell lysate supplemented with iodoacetamide to block free SH groups, treated with endoglycosidase H to simplify the protein patterns on the following gels by removing the N-linked glycans, denatured with SDS and heat in the absence of a reducing agent, and run through a polyacrylamide gel tube. The gel was removed from the tube, and the disulfide-linked proteins were reduced in situ by dithiothreitol treatment. The gel was then loaded on a polyacrylamide slab gel to resolve the reduced monomers in the second dimension (Fig. 3). One half of the endoglycosidase H-treated sample was directly denatured and reduced and loaded onto the second gel for reference (Fig. 3, lane X). The unglycosylated MHBs and SWHs can be seen as 30- and 24-kDa bands, respectively. Proteins which were not disulfide linked in the first-dimension gel were located on a nearly diagonal curve in the gel. This line contained virtually all of the unspecific background label. The originally monomeric MHBs protein can be seen as a prominent spot on this curve. Originally, homodimeric MHBs formed a strong spot at 30

FIG. 3. Disulfide-linked heterodimers between MHBs and SWHs were coexpressed in COS7 cells, labeled for 10 min with radioactive methionine, immunoprecipitated from a Nonidet P-40 cell lysate, treated with endoglycosidase H to simplify the protein pattern, and used for two-dimensional gel electrophoresis. Prior to the first-dimension run, the material was SDS denatured but not reduced. The gel was then treated with dithiothreitol to reduce disulfide bridges in situ and subjected to second-dimension gel electrophoresis to analyze the composition of disulfide-linked oligomers. The weak MHBs and SWHs spots located at identical vertical positions indicate the formation of disulfide-linked MHBs-SWHs heterodimers in the sample. A second aliquot of the sample was directly reduced and loaded on the second gel for reference (lane X). St, molecular mass standard (14.3, 30, 46, and 69 kDa).

kDa below the diagonal. The point of intersection with the diagonal of a vertical line through this spot reveals that the apparent molecular mass of the dimer in the first-dimension gel run, by comparison with the molecular mass standard (Fig. 3, lane St), was approximately 60 kDa, which is in agreement with the expected molecular mass of the homodimer. The heterodimer formed by MHBs and SWHs has a theoretical molecular mass of 56 kDa in the first-dimension gel. On a vertical line at the corresponding position directly to the right of the MHBs dimer, the two spots corresponding to MHBs at 30 kDa and SWHs at 24 kDa can be detected. Theoretically, the proteins in both spots should be present in a one-to-one molar ratio. The stronger signal in the MHBs spot may be explained by the fact that this protein contains seven methionines instead of four, as is the case for SWHs. Also, differences in the rates of synthesis during the short 10-min pulse may account for the different labeling. We deduce from this analysis that the M protein of HBV and S protein of WHV were able to interact directly with each other and form disulfide-linked dimers. The efficiency of heterodimer formation could not be calculated from this experiment because the amount of total SWHs protein was unknown because of the lack of a suitable SWHs-specific antibody.

No incorporation of DHBs proteins into subviral HBV particles. Analogous experiments were performed to study coassembly of the small surface proteins of HBV and DHBV. In this case, the SHBs protein could be used instead of MHBs because the homologous DHBV protein has a molecular mass of 17 kDa and is therefore clearly distinguishable from SHBs on polyacrylamide gels. Furthermore, an antiserum against DHBsAg (anti-DHBs) was available (kindly provided by W. H. Gerlich and S. Lottmann). This antiserum reacted mainly against the pre-S domain of LDHBs and showed only weak binding to SDHBs (19) because of the low immunogenicity of SDHBs (26). In separate transfections, expression and secretion of low amounts of SDHBs by immunoprecipitation with

FIG. 4. No incorporation of SDHBs into subviral SHBs particles. SDHBs (lanes 2, 3, 10, and 11) and SHBs (lanes 4, 5, 12, and 13) were expressed separately or together (lanes 6, 7, 14, and 15) in COS7 cells, pulse-chase labeled, and immunoprecipitated from cell lysates (L) and culture medium (M) with anti-DHBs (lanes 2 to 7) or anti-HBs (lanes 10 to 15). SDHBs was detectable with anti-DHBs (lanes 2, 3, 6, and 7) but was not coimmunoprecipitated with anti-HBs (lanes 14 and 15). By contrast, anti-DHBs showed a weak cross-reaction with SHBs (lanes 4 and 5) but did not coprecipitate larger amounts of SHBs in the coexpressed sample (lanes 6 and 7). Apparently, the small surface protein of avian and human HBV assembled by distinct pathways in the cell into subviral particles and did not mix. St, molecular mass standard (14.3 and 30 kDa).

anti-DHBs (Fig. 4, lanes 2 and 3) and high amounts of SHBs by immunoprecipitation with anti-HBs (Fig. 4, lanes 12 and 13) could be detected. The band at 15 kDa in lane 12 (Fig. 4) was a C-terminal fragment of SHBs generated by internal translation initiation at methionine codon 76 of the HBV S gene. This fragment is only inefficiently incorporated into subviral particles (31). Anti-HBs did not cross-react with SDHBs (Fig. 4, lanes 10 and 11), but anti-DHBs immunoprecipitated low amounts of SHBs (Fig. 4, lanes 4 and 5). This may be due to unspecific interactions with SHBs because the SHBs protein was expressed in high amounts (compare with Fig. 4, lanes 12 and 13) and the autoradiogram was overexposed for SHBs to detect the SDHBs protein. Anti-DHBs did not precipitate more SHBs in coexpression experiments (Fig. 4, lanes 6 and 7) than from material containing only SHBs (Fig. 4, lanes 4 and 5). Likewise, anti-HBs was not able to coprecipitate SDHBs from this material (Fig. 4, lanes 14 and 15). We conclude from this analysis that although the small surface proteins of HBV and DHBV showed a substantial degree of homology and structural similarity (Fig. 1A), they did not mix with each other. Rather, they assembled by separate pathways to distinct subviral particles. The same effect was seen by coexpression of MHBs and SDHBs (data not shown), which excludes the possibility that the different behaviors of SWHs and SDHBs were due to the different HBV envelope proteins used in the coassembly experiments.

Moreover, the inability of DHBV and HBV envelope proteins to mix is shown in Fig. 5. SHBs was coexpressed with LDHBs, which could be detected much better by the anti-DHBs antiserum. Two LDHBs proteins with molecular masses of 35 and 37 kDa were bound to anti-DHBs (Fig. 5, lane 2). Both proteins are not glycosylated (19) and probably represent differently phosphorylated forms of the protein (13). The proteins were not released from the cells (Fig. 5, lane 3), indicating that LDHBs secretion is dependent on the presence of

FIG. 5. No incorporation of LDHBs into subviral SHBs particles. The experiment (lanes 1 to 17) was performed as described in the legend to Fig. 4, lanes 1 to 17, but with LDHBs instead of SDHBs. The coimmunoprecipitation of SHBs by anti-DHBs (lanes 6 and 7) is due to an unspecific association of separate LDHBs material to SHBs particles (compare with lanes 20 and 21, in which separately expressed material was mixed and then immunoprecipitated with anti-HBs) or to a very inefficient incorporation of LDHBs into subviral particles formed by the large excess of SHBs in this sample (compare lanes 14 and 15) because the LDHBs protein was not coimmunoprecipitated with anti-HBs (lanes 14 and 15). LDHBs did not appear in the culture medium (lanes 3 and 7) in a manner analogous to that of the LHBs protein, which needs coassembly with an excess of SHBs to be secreted (compare Fig. 6, lanes 2 and 3) (25).

SDHBs and resembles the secretion behavior of LHBs and SHBs. Immunoprecipitation of coexpressed LDHBs and SHBs with anti-DHBs resulted in clearly visible LDHBs protein but almost no SHBs from the cell lysate (Fig. 5, lane 6), although a large amount of SHBs was present (Fig. 5, lane 14). By contrast, anti-HBs precipitated only the HBV but not the DHBV proteins (Fig. 5, lane 14). In the medium of cotransfected cells, a substantial amount of SHBs was detected with anti-DHBs (Fig. 5, lane 7). We interpret this result as being the consequence of very inefficient incorporation of low amounts of LDHBs into SHBs particles because anti-HBs did not coprecipitate detectable amounts of LDHBs protein from this sample (Fig. 5, lane 15) or, alternatively, as a consequence of unspecific aggregation of low amounts of LDHBs material to SHBs particles because a similar result was obtained when media from separately transfected cultures were mixed and used for immunoprecipitation with anti-DHBs (Fig. 5, lane 21). Taken together, these experiments showed that neither LDHBs nor SDHBs protein was substantially incorporated into 20-nm HBsAg particles.

Stable expression of a WHV pre-S-HBV S chimera. We were also interested in studying phenotypic mixing of LWHs and SHBs protein during subviral particle morphogenesis. For unknown reasons, we were not able to detect the LWHs protein in COS7 cells. Several approaches, like use of an anti-WHV pre-S1 peptide antiserum (kindly provided by H. Will), coexpression with SHBs and coprecipitation with anti-HBs, replacement of the SV40 early promoter by a cytomegalovirus promoter to drive LWHs synthesis, or alteration of the context of the LWHs gene initiation codon according to the rules of Kozak (18), did not result in a detectable band on polyacrylamide gels. In order to study the influence of the WHV pre-S sequence on particle formation, we constructed a chimera referred to as WH which represents the exact fusion of the WHV pre-S1 and pre-S2 sequence to the HBV S sequence (Fig. 1B).

FIG. 6. Stable expression of the WHV pre-S1-pre-S2 HBV S fusion product WH. COS7 cells were transfected with the expression plasmids for the HBV surface proteins (lanes 2 and 3) and for the WHV pre-S1-pre-S2 HBV S fusion (lanes 4 and 5) or mock transfected (lanes 6 and 7) and pulse-chase labeled. Proteins were immunoprecipitated with anti-HBs from cell lysates (L) and culture medium (M) and visualized. As expected, expression of LHBs together with MHBs and SHBs inhibited secretion of the surface proteins (compare, e.g., with Fig. 5, lanes 12 and 13) (25). The fusion construct, however, did not inhibit SHBs secretion and was only detectable in the culture medium (lane 5).

Expression of this construct by a pulse-chase experiment in COS7 cells showed a stable WH product in the medium and, in addition, the WHV pre-S2-HBV S chimera and the SHBs protein (Fig. 6, lane 5). This demonstrated that the MWHs-SWHs promoter located in the WHV pre-S1 sequence was functioning in COS7 cells. Surprisingly, the WH protein was not detectable in cell lysates (Fig. 6, lane 4), which may be explained by an instability of the intracellular protein. In addition, a special feature of the LHBs protein, namely the inhibition of SHBs secretion (25), was not expressed by the WH chimera (compare SHBs bands in Fig. 6, lanes 2 and 3, with bands in Fig. 6, lanes 4 and 5). From this experiment, we could not decide whether LWHs and SHBs form mixed particles because both proteins carry the main HBsAg epitope in their S domains. However, the experiment demonstrated that the WH construct, which was valuable for studying virion formation (see below), was stably expressed.

Incorporation of a DHBV pre-S-HBV S chimera into subviral HBV particles. In a manner analogous to that for the WH construct, we generated a chimera named DH consisting of the DHBV pre-S sequence and the HBV S sequence (Fig. 1B) to study the influence of the DHBV pre-S sequence domain on subviral particle formation and the capability of the DHBV pre-S sequence to function in HBV morphogenesis independently of the DHBV S domain. The formation of mixed subviral particles together with the SHBs protein was tested again by coexpression and coprecipitation in pulse-chase-labeled COS7 cells (Fig. 7). The DH fusion could be detected by immunoprecipitation with anti-HBs (Fig. 7, lane 2) and anti-DHBs (Fig. 7, lane 10) as expected and was only inefficiently secreted (Fig. 7, lanes 3 and 11). This seems to reflect the inhibitory effect of the DHBV pre-S sequence on protein secretion (compare Fig. 4, lane 3 or 7). Coexpression with SHBs resulted in mixed aggregates or particles because the SHBs protein was efficiently coprecipitated by anti-DHBs (compare

FIG. 7. Incorporation of the DHBV pre-S-HBV S fusion construct DH into subviral SHBs particles. The experiment was performed as described in the legend to Fig. 5, except with the fusion construct DH instead of LDHBs. Coimmunoprecipitation of SHBs with anti-DHBs (lanes 6 and 7) and also LDHBs with anti-HBs (lanes 14 and 15) indicated the formation of mixed particles.

Fig. 7, lanes 6 and 7, with Fig. 7, lanes 14 and 15). SHBs was not coprecipitated by anti-DHBs when both proteins were mixed after being expressed in different cells (Fig. 7, lane 17), excluding unspecific association. The SHBs protein from the separately synthesized and subsequently mixed cell lysates which bound to anti-DHBs (Fig. 7, lane 16) was probably expressed from the DH construct (compare with Fig. 7, lane 2). These experiments showed that the DH construct was stably expressed and folded in a way that allowed this protein to be incorporated into subviral 20-nm HBsAg particles probably by the interaction of the DH S domain with the identical SHBs proteins.

Substitution of hepatitis B virion surface proteins by homologous proteins from WHV but not DHBV. Morphogenesis of hepatitis B virions requires the participation of SHBs and LHBs but not MHBs protein (2). Therefore, transfection into the human hepatoma cell line HepG2 of HBV genomes carrying a stop codon in the pre-S1 sequence (L^-) or a missense mutation of the SHBs start codon (S^-) does not lead to production of virions. However, this defect can be complemented in *trans* by coexpressing the missing HBV surface protein (2). We made use of this system and tested by cotransfection experiments whether foreign hepadnavirus surface proteins were able to substitute for SHBs and LHBs in the virion envelope. Secretion of virions from transfected HepG2 cells was monitored by immunoprecipitation, radioactive labeling of the viral genome by the endogenous polymerase of the virus, and detection of the isolated genome by agarose gel electrophoresis and autoradiography (2) (Fig. 8). Four immunoprecipitations were done for each assay (lanes from left to right in each panel of Fig. 8): (i) from the culture medium with an antiserum against the HBV nucleocapsid (anti-HBc) in the absence and (ii) presence of a mild detergent to determine the amount of naked and total nucleocapsids, respectively; (iii) with anti-HBs to test for the presence of HBV surface protein in the outer shell of the virions; and (iv) from Nonidet P-40 cell lysates with anti-HBc to detect cytoplasmic nucleocapsids as a reference.

The HBV genome lacking a functional S gene could be complemented in *trans* as expected by expressing the autologous SHBs protein and also by SWHBs but not by SDHBs (Fig.

FIG. 8. Complementations of HBV genomes defective in expressing SHBs or LHBs protein by homologous surface proteins of animal hepadnaviruses. HepG2 cells were transfected with a replication-competent HBV genome carrying a missense mutation of the S gene start codon suppressing SHBs synthesis (A) or a stop codon in the pre-S1 sequence (B and C) suppressing LHBs expression. Both proteins are necessary to form virions (2). Virions were detected in each panel by immunoprecipitation (from left to right) from the culture medium (i) with anti-HBc in the absence and (ii) with anti-HBc in the presence of mild detergent and (iii) with anti-HBs and (iv) with anti-HBc from cell lysates followed by a radioactive endogenous polymerase reaction. (A) The defect of the S^- HBV genome could be complemented in *trans* by the autologous SHBs and by SWHBs but not by SDHBs. (B) Virion formation of the L^- HBV genome was restored by the autologous LHBs but not by LDHBs. The lanes marked by asterisks represent immunoprecipitations of the medium with anti-DHBs. (C) The L^- genome could be weakly but reproducibly complemented by the WH but not by the DH fusion construct. The experiments show that the surface protein domains of WHV but not of DHBV were able to functionally substitute for the homologous protein domains of HBV in virion morphogenesis.

8A). Furthermore, the L^- HBV genome could be complemented by LHBs but not LDHBs (Fig. 8B). The lanes marked with an asterisk represent additional immunoprecipitations with anti-DHBs. Virion formation by the L^- HBV genome was weakly but detectably restored by coexpression of the WH chimera but not by the DH chimera (Fig. 8C).

Taken together, the results on the formation of mixed subviral particles and substitution of surface proteins in the virion envelope agree with each other: the WHV S protein and pre-S chimera were not only embodied in subviral particles but were also able to functionally substitute for the homologous protein or protein domain of HBV in virion formation. The SDHBs and LDHBs proteins, on the other hand, were neither incorporated into subviral particles nor able to participate in hepatitis B virion morphogenesis.

DISCUSSION

By coexpression and coimmunoprecipitations with species-specific antisera, we could show that the middle surface protein of HBV and the small surface protein of WHV assemble to form mixed subviral particles (Fig. 2). Furthermore, the SWHs protein and the WHV pre-S domain fulfilled the essential functions of their homologous HBV counterparts in hepatitis B virion morphogenesis (Fig. 8). Such a complementation by orthohepadnavirus S proteins in which 76% of the S gene of the ground squirrel hepatitis B virus was replaced by the homologous region from WHV without destroying the infectivity of the recombinant has also been described by Seeger et al. (27). Phenotypic mixing has even been observed between the main structural nucleocapsid proteins of WHV and HBV (5), although nucleocapsid protein mixing is much more restricted than envelope protein mixing (9). Furthermore, a defect in the polymerase gene of HBV could be complemented in *trans* by the polymerase gene from WHV but not from DHBV in a cell transfection assay (23). This demonstrates the structural similarity of WHV and HBV. On the other hand, the avihepadnavirus surface proteins from DHBV were not incorporated into HBV particles (Fig. 4, 5, and 8). Rather, SHBs and SDHBs or LDHBs assembled by separate pathways after expression in the same cells (Fig. 4 and 5). Phenotypic mixing of the nucleocapsid proteins (5) or *trans*-complementation of the HBV polymerase gene by the DHBV gene (23) was also not possible. Our result was nevertheless surprising to us because phenotypic mixing of envelope proteins from virus families as different as retroviruses and rhabdoviruses has been observed (33), whereas phenotypic mixing of nucleocapsid components is less common. In our case, the small surface protein of DHBV even shows a 23 to 38% amino acid identity and clear structural similarity with its HBV counterpart (Fig. 1A). We deduce from this that the assembly of hepadnavirus particles requires relatively precise molecular interactions of the surface proteins which are conserved between two members of the same genus but not within this virus family.

The appearance of mixed SWHs-MHBs particles (Fig. 2, lanes 5 and 6) does not prove that both proteins mix without preference. It is possible, for example, that two different kinds of particles were generated: one kind consisting of SWHs with a very low amount of MHBs incorporated and vice versa. The low amount of MHBs in the SWHs particles may be sufficient for immunoprecipitation, and the net result would be like that shown in Fig. 2. We therefore tried to demonstrate the interaction between both proteins more directly. This was possible because both proteins formed disulfide-linked heterodimers (Fig. 3). Although we cannot precisely calculate how well both proteins interact because we could not determine the total amount of SWHs expressed by the cells, the experiments showed that such a covalent interaction is observable. Our experiments (31) and other studies (21) on SHBs cysteine mutants revealed that the efficiency of secretion of subviral SHBs particles depends on cysteine residues 48, 65, 69, 107, 138, and 149 (numbering starts with the N terminus of SHBs). All these cysteines are conserved in the WHV sequence. The remaining seven cysteine residues of SHBs at positions 76, 121, 124, 136, 137, 147, and 221 are dispensable for subviral particle formation (31). Only three of these cysteines are conserved in SWHs. The SDHBs sequence, on the other hand, contains only three cysteine residues at positions 48, 65, and 69, which are located in the cytosolic loop between translocation signals I and II (Fig. 1B). The lack of further cysteine residues in SDHBs at positions homologous to positions 107, 138, and 149 of SHBs in the luminal loop downstream of signal II might be

one reason why the formation of mixed SHBs-SDHBs dimers in the ER membrane could not be observed (Fig. 4, lanes 6 and 14). The DH fusion construct revealed that coassembly into subviral particles and virions could be uncoupled. The S domain was apparently sufficient to guide this protein to the pathway for subviral particle morphogenesis (Fig. 7, lanes 7 and 15). Such an effect of the S domain has also been shown with a globin-SHBs fusion (1). However, the DH construct could not restore the L⁻ defect of an HBV genome (Fig. 8C). If the model is correct that the pre-S domain of L has the function of a matrix protein (3, 4, 24), our result would suggest that the DHBV pre-S domain is unable to trigger the interaction with the HBV nucleocapsid necessary for virion morphogenesis. This is in agreement with the lack of a sequence homology of HBV and DHBV pre-S sequences (Fig. 1A).

The L protein of HBV is initially synthesized as a transmembrane protein with a cytosolic pre-S domain. During maturation, approximately half of the molecules change their folding in such a way that the pre-S domains become exposed on the outer surface of virions (3, 24). The DHBV L protein also shows a dual topology in viral particles (29a). Secreted mixed particles consisting of DH and SHBs could be immunoprecipitated with anti-DHBs (Fig. 7, lane 15) almost as efficiently as with anti-HBs (Fig. 7, lane 7), which showed that the DHBV pre-S domain was exposed on the surfaces of these particles. Since the DHBV pre-S sequence does not contain a stretch of hydrophobic amino acids typical for translocation signals, we assume that the DHBV pre-S domain in DH is first located at the cytosolic side of the ER membrane and performs a topology switch during mixed particle assembly analogous to that for LHBs. Interestingly, a globin-S fusion could be incorporated into SHBs particles only when the globin domain was cotranslationally translocated into the lumen of the ER by the additional fusion of an N-terminal signal sequence (1).

The WHV pre-S domain was able to partially substitute for the HBV pre-S domain in virion formation (Fig. 8C). By deletion mapping, a C-terminal part of pre-S starting with amino acid 103 has been shown to be indispensable for virion morphogenesis (4). This part of HBV shows the highest homology within the pre-S sequence to that of WHV (Fig. 1A). More precisely, the HBV pre-S1 sequence 103 to 119 contains 8 identical (47%) or 11 similar (65%) amino acids relative to that of WHV, which carries, however, a 29-amino-acid insertion in this region. The N-terminal part of pre-S is much less conserved (Fig. 1A). This is consistent with the idea that the C-terminal region of pre-S functions as a matrix protein.

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