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The role of envelope proteins in hepatitis B virus assembly

(viral assembly/membrane proteins)

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ABSTRACT **Hepatitis B virus (HBV) particles are generated by budding of preformed cytoplasmic nucleocapsids into endoplasmic reticulum (ER) membranes containing the three viral envelope proteins (L, M, and S). We have examined the contributions of the envelope proteins to virion assembly by using cultured hepatoma cells transfected with mutant HBV genomes bearing lesions in the envelope coding regions. We show here that HBV nucleocapsids are not released from cells without expression of envelope proteins, implying an active role for these proteins in viral morphogenesis. S and L but not M proteins are necessary for virion production. L protein overexpression inhibits virion release, just as it inhibits the release of subviral hepatitis B surface antigen (HBsAg) particles. Mutant L proteins that are no longer capable of retaining HBsAg particles in the ER still allow virion formation, indicating that this ER retention reaction is not required for viral budding. Myristoylation of L protein is also dispensable for virion formation. A chimeric protein bearing foreign epitopes fused to the S protein can be incorporated into virions when coexpressed with the wild-type envelope proteins. Models for the dependence of virion formation on both L and S proteins are discussed.**

Hepatitis B virus (HBV) is an enveloped DNA virus of the hepadnavirus family. Its partially duplex, open circular DNA genome is encapsidated in a nucleocapsid together with a virus-encoded polymerase; this core particle is surrounded by a lipid envelope bearing the viral surface glycoproteins (1–3). Little is known about the morphogenesis of HBV virions (also called Dane particles), but several features indicate that this process is likely to be both complex and distinctive.

First, the envelope proteins of HBV are unusually complex. Virions contain three distinct but related surface proteins (termed L, M, and S) produced by alternative translation initiations in a single long open reading frame (refs. 4 and 5; cf. Fig. 1*B*). This generates a family of proteins, all of which harbor a common 24-kDa C-terminal domain (S); the two larger proteins (M and L) contain N-terminal extensions of 55 and 174 amino acids, respectively (see Fig. 1*B*). Each of these polypeptides is cotranslationally inserted into the endoplasmic reticulum (ER) membrane (6, 7). But unlike other envelope proteins, which have single transmembrane domains, the surface proteins of HBV span the bilayer multiple times (8, 9). The L protein is further modified by myristoylation (10). Finally, unlike all other envelope proteins the HBV surface proteins can also be independently secreted from cells as subviral particles lacking all other viral components (11). These subviral particles are generated within the ER lumen by a nucleocapsid-independent process involving extrusion of the envelope proteins from the ER membrane (12–14). Dane particles are generated by budding of preformed cytoplasmic nucleocapsids into the ER (15); the resulting virions are then exported from the cell via the constitutive pathway of vesicular transport (16).

These observations raise many interesting questions about HBV assembly. Are all of the viral surface proteins required for assembly, or are some specialized to carry out other roles in the life cycle (e.g., host cell binding)? How does virion assembly differ from the assembly of subviral particles? What regulates the flow of envelope proteins into these two morphogenetic pathways? To explore these and other questions we have developed a convenient assay for Dane particle formation and have used it to study virion assembly in permissive HepG2 cells (2) transfected with mutant viral genomes encoding altered envelope proteins.

MATERIALS AND METHODS

Plasmid Constructions. Plasmid pHBV1.5 contains an overlength (1.5-mer) copy of HBV DNA, subtype adw (5), inserted into pBLUESCRIPT KS(+) (Stratagene). The 5′ end of the insert is at the *Eco*RV site [nucleotide (nt) 1040]; the 3′ end is at the *Bsp*MII site (nt 2327). For the construction of pHBV1.5myr$^-$, a 600-base-pair preS1 fragment was amplified by a polymerase chain reaction from the plasmid pSV45m1m2 (17); the upstream primer in this polymerase chain reaction restored the *Bst*EII site (nt 2814), which was lost during the construction of pSV45m1m2. The amplified fragment was digested with *Bst*EII and *Eco*RI (nt 1) and inserted into *Bst*EII/*Eco*RI-digested pHBV1.5. The inserted fragment was sequenced and found to be wild type (WT) except for the two point mutations changing the 2nd and 13th codons of the preS1 region from glycine to alanine. Plasmids pSV45H and pSV24H, both subtype adw, have been described (17, 18). They contain the whole preS1–preS2–*S* gene (pSV45H) and the *S* gene alone (pSV24H) under the transcriptional control of the simian virus 40 (SV40) early promoter. pSV45y carries the preS1–preS2–*S* gene of HBV subtype ayw under control of this SV40 promoter. pSVsGS has an in-frame fusion of the β-lactamase signal sequence to the first 100 codons of the chimpanzee β-globin gene; this in turn is fused to codon 44 of the preS2 region (6, 8).

For generating the env$^-$ and S$^-$ mutations a 1-kilobase *Eco*RI (nt 1)/*Nsi* I (nt 1066) fragment containing the *S* gene was inserted into the phagemid vector pGC2. Single-stranded DNA was prepared from the *dut unc* mutant *Escherichia coli* CJ236 (Bio-Rad) by infection with the helper phage R408. For construction of the L and M mutants, single-stranded DNA was prepared from a M13 derivative containing a 647-nt fragment of the HBV genome encoding the preS1 and preS2 region (17), which was grown in *E. coli* CJ236. *In vitro* mutagenesis was performed according to Kunkel *et al.* (19). The following oligonucleotides were used to introduce the

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Abbreviations: HBV, hepatitis B virus; ER, endoplasmic reticulum; nt, nucleotide(s); WT, wild type; SV40, simian virus 40; EPR, endogenous polymerase reaction; HBs, hepatitis B surface protein; HBc, hepatitis B core protein; NP-40, Nonidet P-40.

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indicated mutations: env$^-$, 5′-CCTGCTCGTGTG*ACAGGCGGG-3′; S, 5′-GTGACGAACAC*GGAGAACATC-3′; M$^-$, 5′-CCTCAGGCCAC*GCAGTGGAATTCC-3′; L$^-$, 5′-CAAGGACCACTA*GCCAGCAGCC-3′ (* denotes mutation). Mutants were identified directly by sequencing and were then transferred back into the pHBV1.5 background.

Immune Precipitation and Endogenous Polymerase Reaction (EPR). Polyclonal goat anti-HBs, rabbit anti-HBc, and rabbit anti-globin antibodies were from Dako (Santa Barbara, CA). Immune precipitations were carried out as described (26). For EPR, 50 μl of a mixture containing 50 mM Tris·HCl (pH 7.5), 75 mM NH_4Cl, 1 mM EDTA, 25 mM $MgCl_2$, 0.1% 2-mercaptoethanol, 0.5% Nonidet/P-40 (NP-40), 0.4 mM each dGTP, dATP, and dTTP, 10 μCi [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) was added to each immune precipitate and incubated overnight at 37°C. The DNA was then isolated by digestion with proteinase K (Sigma) at 300 μg/ml for 30 min at 37°C, followed by a phenol/chloroform and a chloroform extraction and two ethanol precipitations. The Sepharose beads concentrated at the interphase of the extraction. The DNA was separated on a 1% agarose/1× TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) gel, which was dried before autoradiography.

RESULTS

Experimental System. To study virion assembly in HBV-transfected HepG2 cells, a simple but powerful two-step protocol was used. The first step consisted of an immune precipitation of virus-specific particles from the medium using antibodies against the major surface protein (anti-HBs) or against the viral core (anti-HBc). To detect particles bearing HBV genomes (i.e., virions or nucleocapsids), a second step, consisting of the EPR, was carried out. In this reaction, labeled dNTPs are incorporated into the plus strands of encapsidated viral DNA (1); this DNA is then extracted and analyzed by agarose gel electrophoresis. Thus, in this assay the signature of a HBV virion is the precipitation of labeled HBV DNA by anti-HBs but not anti-HBc; after removal of the viral envelope with detergent (NP-40), viral DNA is precipitated by anti-HBc but not anti-HBs.

HepG2 cells were transfected with the WT cloned HBV genome (pHBV1.5) shown in Fig. 1*A*. Five days later, medium and cytoplasmic extracts were harvested and immune precipitated with anti-HBc in the presence of NP-40. The detergent was added to disrupt the virion envelope so that in one step the total amount of EPR-reactive cores in the medium and cells could be detected. As shown in Fig. 2 (lanes b and e) the EPR labeled two DNA species in both preparations, corresponding to the open circular and linear forms of the HBV genome. We next precipitated the medium with anti-HBc without adding detergent to test whether extracellular cores were present in a naked or enveloped form. As shown in lane a, almost no naked nucleocapsids were detectable. By contrast, the anti-HBs antibody coprecipitated as much viral genome in the absence of detergent as the anti-HBc antibody did in the presence of detergent (compare lanes c and b). From this we conclude that the cells released EPR-competent cores almost exclusively as properly enveloped virions.

If viral genomes are exported exclusively as virions, precipitation of viral DNA should not occur with anti-HBs after detergent treatment. Fig. 2 (lane d) shows that this is indeed the case. Interestingly, however, this required that reducing agent (30 mM dithiothreitol) be added to the immune precipitation reaction. If dithiothreitol is omitted, virtually all the viral cores are precipitated by anti-HBs even in the presence of 0.5% NP-40 (data not shown). This suggests that the envelope proteins form a disulfide-linked adherent network that cannot be removed from the virions by detergent alone; perhaps some surface proteins might be linked to the nucleocapsid by disulfide bridges.

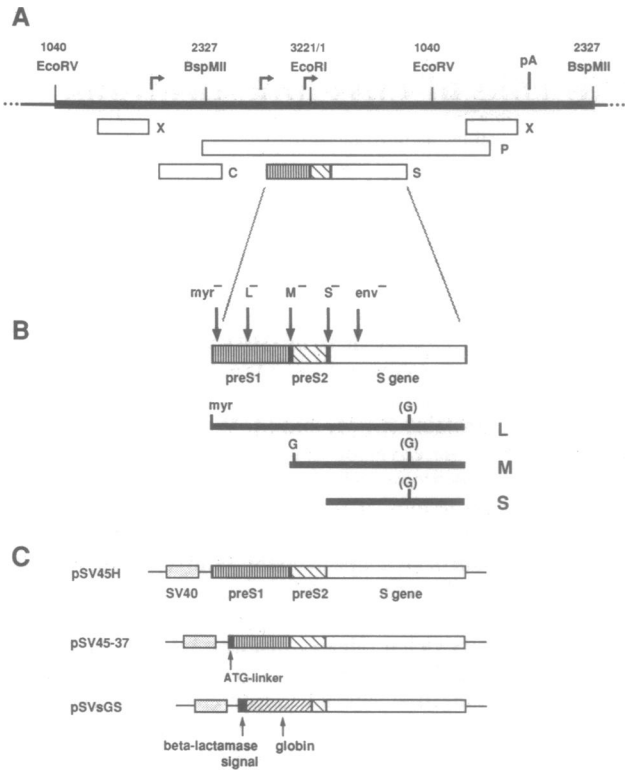

FIG. 1. Plasmids encoding normal and mutant HBV envelope proteins. (*A*) HBV open reading frames (ORFs) included in plasmid pHBV1.5. Heavy line, HBV sequences; thin line, plasmid sequences; boxes, ORFs for viral X, P, C, and S proteins. (*B*) The envelope ORF and its products. Box, ORF divided into its three subregions (known as preS1, preS2, and S) by in-frame AUG codons (dark vertical bars) used to initiate translation of the L, M, and S proteins, respectively; these proteins are depicted as horizontal bars below the ORF. G, position of N-linked glycans; myr, covalently linked myristate. Above the ORF is shown the location of the L and env stop codon mutations, and the myr, M, and S missense mutations. (*C*) Complementing plasmids. pSV45 supplies L protein from the SV40 early promoter (stippled box); M and S proteins are transcribed from HBV promoter within preS DNA. In pSV45-37, the N-terminal 30 codons of preS1 are replaced by a synthetic linker providing a new AUG. pSVsGS encodes a globin–HBV fusion protein bearing a signal sequence from *E. coli* β-lactamase.

Nucleocapsid Release Requires Surface Proteins. To test whether the release of cores from the host cell is dependent on the expression of surface proteins, cells were transfected with a mutant plasmid, pHBVenv$^-$, which has a single point mutation changing the 15th codon of the *S* gene from TTA to the stop codon TGA. This mutation is silent in the overlapping *P* gene and is expected to direct only the synthesis of truncated, nonfunctional surface proteins (Fig. 1*B*). With this mutant, we detected about the same amount of cores in the cell lysate (Fig. 2, lane j) as in WT (lane e), but almost no virions were present in the medium (lanes f–i). To confirm that this phenotype is due to the absence of functional surface proteins, we cotransfected the env$^-$ mutant together with the expression vector pSV45H, which supplies WT L, M, and S proteins (Fig. 1*C* and legend). pSV45H efficiently complemented the env$^-$ mutation, resulting in the release of virions (lanes k–n).

To determine the dependence of virion release on each of the three envelope proteins individually, we constructed three mutants that selectively abolish the expression of either the L, M, or S proteins (Fig. 1*B*) without changing the coding potential of the overlapping *P* gene. The L$^-$ mutation creates

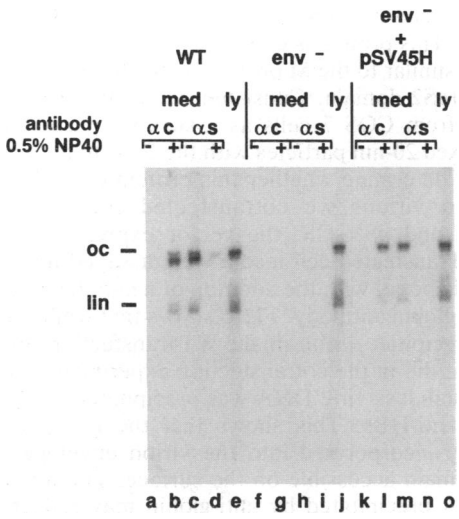

FIG. 2. HBV virion assembly requires envelope proteins. HepG2 cells were transfected by pHBV1.5 (WT; lanes a–e), pHBVenv$^-$ (env$^-$; lanes f–j), or pHBV env$^-$ plus pSV45H (lanes k–o). Five days later, media (med) were harvested and immunoprecipitated with anti-HBc (αc) or anti-HBs (αs) in the presence (+) or absence (−) of NP-40 pretreatment, as indicated above each lane. Cytoplasmic lysates (ly; lanes e, j, and o) were precipitated only with anti-HBc. After labeling by the EPR, labeled viral DNA was extracted and analyzed by 1% agarose gel electrophoresis and autoradiography. oc, Open circular viral DNA; lin, linear viral DNA.

a stop codon at codon 52 of the preS1 region. The M$^-$ and S$^-$ mutations are point mutations that change the translation initiation codons for M and S proteins, respectively, from ATG to ACG. The M$^-$ mutation abolishes M protein expression and changes a methionine residue in the L protein to threonine. The S$^-$ mutant ablates S protein expression and changes a methionine residue in the M and L polypeptides to threonine. The assembly phenotype of the mutants is shown in Fig. 3*A*. All three mutants produced cytoplasmic cores with WT efficiency (lanes e, j, and o). While the M$^-$ mutation still allowed virion secretion (lanes f–i), the S$^-$ and L$^-$ mutations abolished virion release (lanes a–d and k–n). Complementation of L$^-$ with pSV45H restored WT behavior, as did complementation of the S$^-$ mutant with pSV24H, which expresses WT S protein (data not shown). Thus, L and S but not M proteins are necessary for virion morphogenesis; the complementation studies with pSV24H also indicate that the missense changes in the preS proteins encoded by the S$^-$ mutant do not impair assembly.

L Protein Myristoylation and Virion Release. A prominent feature of the L protein is the posttranslational modification of its N-terminal glycine residue with myristic acid (10). We constructed a mutant (pHBVmyr$^-$) that encodes a nonmyristoylated L protein to test the significance of this modification for virion maturation. The L gene of the adw serotype encodes two Met-Gly dipeptides in its first 14 codons; both can serve as myristate acceptors, although the first predominates because of preferential translation initiation at the first methionine codon. Accordingly, we changed both glycine codons (GGA and GGG) to alanine codons (GCA and GCG, respectively); these two point mutations are silent in the *P* gene and have been previously shown to abolish myristoylation of the L protein completely (17). The assembly phenotype of the myr$^-$ mutant was indistinguishable from WT (Fig. 3*B*), showing that the myristoylation of the L protein is dispensable for virion morphogenesis. The same result was obtained with a myr$^-$ mutant of the HBV ayw subtype, which contains only one potential myristoylation signal (data not shown).

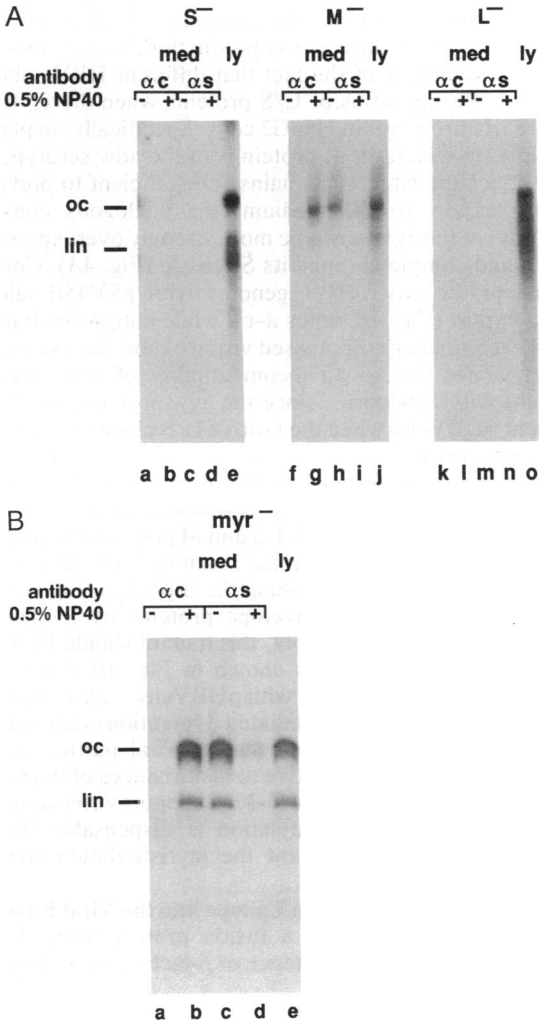

FIG. 3. (*A*) Virion export requires S and L but not M proteins. HepG2 cells were transfected with pHBV1.5 derivatives bearing the S (lanes a–e), M (lanes f–j), or L (lanes k–o) mutations. Five days later, medium (med) was harvested and precipitated with anti-HBc (lanes a, b, f, g, k, and l) or anti-HBs (lanes c, d, h, i, m, and n) in the presence (+) or absence (−) of NP-40 as indicated. Cytoplasmic lysates were precipitated only with anti-HBc. (*B*) L protein myristoylation is not required for virion formation or release. Media (med; lanes a–d) from cells transfected with pHBV1.5 myr$^-$ were precipitated with anti-HBc (lanes a and b) or anti-HBs (lanes c and d) in the presence (+) or absence (−) of NP-40, as indicated above each lane; cell lysates (ly; lane e) were precipitated with anti-HBc only. After EPR labeling of the precipitated viral genomes, viral DNA was analyzed by agarose gel electrophoresis as in Fig. 2. oc, Open circular viral DNA; lin, linear viral DNA.

Effect of L Protein Overproduction on Virion Release. One special feature of HBV is that surface proteins are released from the cell not only as virions but also as subviral lipoprotein particles. These contain large quantities of S polypeptides, variable amounts of M protein, and only trace amounts of L chains (1, 4). Overexpression of L protein inhibits the release of these subviral particles (18, 20, 21) and it has been shown that the N-terminal 19 amino acids of the L polypeptide are responsible for this intracellular retention (17). Retained envelope proteins accumulate in the ER. It has been speculated (18) that one function of the L protein in virion morphogenesis might be to generate patches of retained HBV envelope proteins in the ER membrane by forming transmembrane aggregates with M and S proteins. These patches could then become sites for viral budding, since in these sites all three envelope proteins would be concentrated together.

If so, then augmenting the amount of retained envelope proteins might enhance virion production. To test this idea, we took advantage of the fact that different HBV subtypes express differing ratios of L/S proteins when driven by the SV40 early promoter in HepG2 cells. Specifically, in plasmid pSV45H, in which the L protein is of the adw serotype, the ratio of accumulated L/S chains is insufficient to prevent S protein export to the medium; the analogous construct (pSV45y) of the ayw serotype more strongly overexpresses L chains and completely inhibits S release (Fig. 4*A*). Cotransfection of the env$^-$ HBV genome with pSV45H allowed virion export (Fig. 4*B*, lanes a–e), while cotransfection with pSV45y completely suppressed virion export (lanes f–j). This defect is not due to an incompatibility of ayw envelope proteins with adw cores, since the ayw proteins could complement pHBVenv when the ratio of L/S chains was lowered (data not shown).

Another way to test the significance of envelope protein retention by L chains was to cotransfect pHBVenv$^-$ with pSV45-37, which encodes WT S and M proteins but a mutant L protein missing the N-terminal 39 amino acids. This mutant is no longer capable of retaining the S protein (Fig. 4*A*; ref. 17). If ER retention of envelope proteins by L chains is important for virion assembly, this mutant should be unable to produce virions. But as shown in Fig. 4*B* (lanes k–o), coexpression of this mutant with pHBVenv$^-$ allowed normal virion secretion. Thus, L-mediated S retention is not required for virion production; virion and subviral particle release appear to be similarly sensitive to disturbances of the normal L/S ratio. The ability of pSV45-37 to support virion assembly also confirms that myristoylation is dispensable for this activity, since in this mutant the myristoylation signal is entirely deleted.

Incorporation of a Foreign Epitope into the Viral Envelope. Recently, we constructed a fusion protein (termed sGS) consisting of the signal sequence of β-lactamase and the first 100 amino acids of chimpanzee α-globin fused to the S protein (Fig. 1*C*). This protein is expected to have a transmembrane topology similar to the M protein, with globin largely replacing the preS2 domain. Consistent with this, it is efficiently secreted from COS 7 cells as a subviral particle and also forms mixed 20-nm particles with the S protein (unpublished data). To determine whether this protein could be incorporated into virions we cotransfected HepG2 cells with pHBV1.5 and pSVsGS, the vector expressing the fusion protein. Transfected cell media were assayed for virions as described above, with the addition of a parallel precipitation with anti-globin antibody. Fig. 5 shows that while anti-globin did not precipitate virions in the WT transfection alone (lanes e and f), it did in the cotransfection experiment (lanes l and m), although less viral DNA was precipitated by anti-globin than by anti-HBs. This shows that the fusion protein is detectably incorporated into the virion envelope, with its globin domain accessible on the surface. The low levels of viral DNA precipitated by anti-globin may reflect a lower titer or affinity of this antiserum compared to antiHBs or reduced efficiency of assembly of sGS into the viral envelope (or both).

DISCUSSION

These data show that HepG2 cells transfected with HBV DNA secrete mainly enveloped virions and almost no naked nucleocapsids, as judged by the fact that all EPR-competent cores were precipitated by anti-HBs rather than anti-HBc (Fig. 2). We cannot exclude that naked cores that are not competent for the EPR may have been secreted. [In occasional experiments, we did observe low-level release of naked cores, as has been found by others (2); it is our impression that this is most likely due to cell damage caused by the transfection procedure.] The finding that HBV cores are not released from the cell without expression of envelope

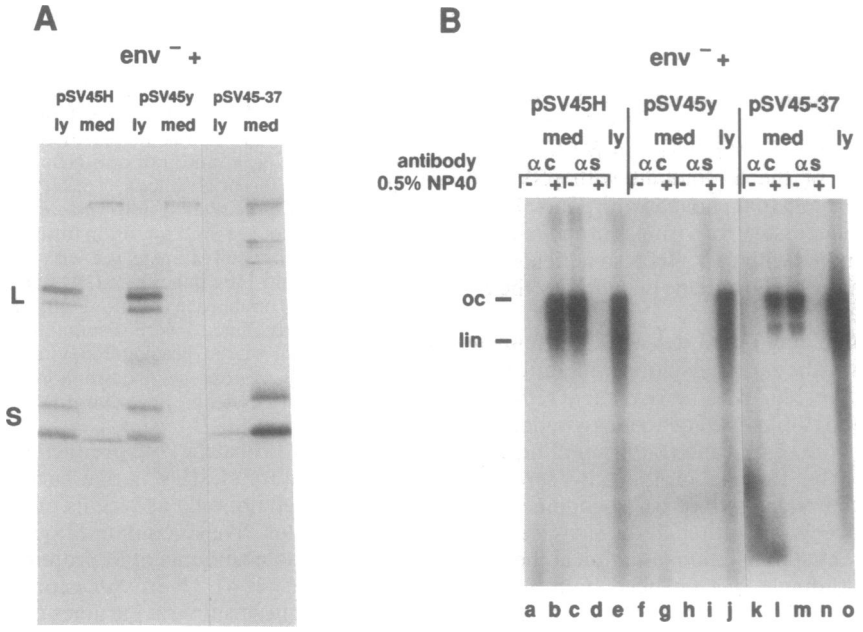

FIG. 4. (*A*) L protein overexpression inhibits surface antigen export. HepG2 cells were transfected with pHBVenv$^-$ plus either pSV45H, pSV45y, or pSV45-37, as indicated above the appropriate lanes, and radiolabeled for 1 hr in 0.6 ml of medium with 0.1 mCi of [^{35}S]methionine. After a 24-hr chase in unlabeled methionine, cell lysates (ly) or media (med) were precipitated with anti-HBs and examined by SDS/PAGE. L, position of the L polypeptide doublet; S, position of the S protein doublet. Doublets arise from the presence of unglycosylated and glycosylated versions of the polypeptides (4). (*B*) L protein overexpression inhibits virion release. In parallel with the experiment of Fig. 5*A*, cells were cotransfected with pHBVenv$^-$ plus either pSV45H (lanes a–e), pSV45y (lanes f–j), or pSV45-37 (lanes k–o); 5 days later, media (med) were precipitated with anti-HBc (lanes a, b, f, g, k, and l) or anti-HBs (lanes c, d, h, i, m, and n) in the presence (+) or absence (−) of NP-40 as indicated above each lane. Lysates (ly) were precipitated only with anti-HBc. Precipitated genomes were labeled by the EPR and analyzed by agarose gel electrophoresis and autoradiography. oc, Open circular viral DNA; lin, linear viral DNA.

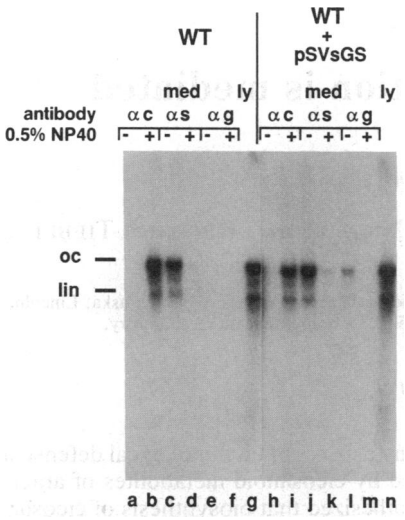

FIG. 5. Incorporation of foreign epitopes on engineered HBV virions. HepG2 cells were transfected with either pHBV1.5 (WT; lanes a–g) or pHBV1.5 plus pSVsGS (lanes h–n). Cytoplasmic lysates (ly; lanes g and n) were harvested 5 days later and precipitated with anti-HBc. Media (med) harvested at this time were precipitated with anti-HBc (lanes a, b, h, and i), anti-HBs (lanes c, d, j, and k) or anti-globin (lanes e, f, l, and m), in the presence (+) or absence (−) of NP-40, as noted above each lane. Precipitated cores were labeled in the EPR and labeled viral DNA was examined by agarose gel electrophoresis and autoradiography. oc, Open circular viral DNA; lin, linear viral DNA.

proteins is in sharp contrast to the situation observed in retroviral assembly, in which nucleocapsids can be exported in the absence of envelope glycoproteins (22). This implies that in HBV budding, surface proteins must interact, directly or indirectly, with core proteins. Precedent for this exists among other enveloped RNA viruses, especially the alphaviruses, where the evidence strongly indicates a direct interaction between nucleocapsid and envelope polypeptides (23).

This work, together with recent studies of Summers *et al.* (24), strongly implicates the L protein as a key regulator of the fate of cytoplasmic core particles. Summers *et al.* have shown that avian hepadnaviral mutants lacking L protein show enhanced transport of viral cores to the nucleus, where their open circular viral DNA is converted to the closed circular form that serves as a template for viral transcription (24). Since L is required for viral release (Fig. 3*A*), a simple model for the control of core transport is suggested: when L levels are low (e.g., after viral uptake and prior to viral gene expression) delivery of incoming cores to the nucleus is favored. As L protein levels increase later in infection, export of cores as mature Dane particles then occurs.

Although M proteins are found on HBV virions (4), they appear to be dispensable for morphogenesis. We assume they are incorporated into the viral envelope via lateral interactions of their S domains with those of the L and S protein (or possibly with core proteins). The ability to incorporate into the envelope a globin–S fusion protein in which preS2 sequences have been largely replaced indicates that these sequences are not essential for incorporation. This result suggests that it may be possible to integrate into the viral membrane other protein domains fused to S in a similar manner. By judicious choice of such foreign domains, it might be possible to engineer hepadnaviral virions with specific biological properties (e.g., altered host range or tissue tropism).

Why is the L protein necessary for virion formation? An early model invoked a role for the L-mediated ER retention of envelope proteins in creating sites for budding (18). But this notion is clearly excluded by our data (Fig. 4); alternative models are required. For instance, L protein might exhibit additional cytoplasmic sequences in its preS1 domain that could function in core recognition. However, epitope mapping studies suggest that most or all of the preS1 domain of L is initially the ER lumen, since (after budding) these sequences are displayed on the particle surface (25). Alternatively, if the only cytoplasmic sequences of the surface proteins are in the S domain, then it might be that the conformation of these sequences in L chains is different from that in S chains, or that interactions with L proteins change the conformation of cytoplasmic domains of S polypeptides during their aggregation.

Finally, L protein myristoylation is not required for Dane particle formation and release. Although not involved in HBV assembly, myristoylation clearly plays some functional role in hepadnavirus biology: this feature is conserved in all viral isolates, and DHBV mutants defective for myristoylation are assembly competent but noninfectious (26). This suggests that myristoylation of hepadnavirus envelope proteins is involved in a subsequent step, perhaps in host cell binding or membrane fusion.

1. Ganem, D. & Varmus, H. E. (1987) *Annu. Rev. Biochem.* **56,** 651–694.
2. Sureau, C., Romet-Lemonne, J.-L., Mullins, J. I. & Essex, M. (1986) *Cell* **47,** 37–47.
3. Dane, D. S., Cameron, C. H. & Briggs, M. (1970) *Lancet* **i,** 695–698.
4. Heermann, K. H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52,** 396–402.
5. Valenzuela, P., Quiroga, M., Zaldivar, J., Gray, P. & Rutter, W. J. (1980) in *Animal Virus Genetics*, ICN–UCLA Symposium on Molecular and Cellular Biology, eds. Fields, B. N. & Jaenisch, R. (Academic, New York), Vol. 18, pp. 57–70.
6. Eble, B. E., Lingappa, V. R. & Ganem, D. (1986) *Mol. Cell. Biol.* **6,** 1454–1463.
7. Eble, B., Lingappa, V. & Ganem, D. (1990) *J. Virol.* **64,** 1414–1418.
8. Eble, B. E., Macrae, D. R., Lingappa, V. R. & Ganem, D. (1987) *Mol. Cell. Biol.* **7,** 3591–3601.
9. Peterson, D. (1987) *BioEssays* **6,** 258–262.
10. Persing, D. H., Varmus, H. E. & Ganem, D. (1987) *J. Virol.* **61,** 1672–1677.
11. Dubois, M. F., Pourcel, C., Rousset, S., Chany, C. & Tiollais, P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4549–4553.
12. Gerber, M., Hadziyannis, S., Vissoulis, C., Schaffner, F., Paronetto, F. & Popper, H. (1974) *Am. J. Pathol.* **75,** 489–502.
13. Patzer, E. J., Nakamura, G. R., Simonsen, G. C., Levinson, A. D. & Brands, R. (1986) *J. Virol.* **58,** 884–892.
14. Simon, K., Lingappa, V. & Ganem, D. (1988) *J. Cell Biol.* **107,** 2163–2168.
15. Roingeard, P., Lu, S., Sureau, C., Freschlin, M., Arbeille, B., Essex, M. & Romet-Lemonne, J. (1990) *Hepatology* **11,** 277–285.
16. Kelly, R. (1985) *Science* **230,** 25–31.
17. Kuroki, K., Russnak, R. & Ganem, D. (1989) *Mol. Cell. Biol.* **9,** 4459–4466.
18. Persing, D., Varmus, H. & Ganem, D. (1986) *Science* **234,** 1388–1392.
19. Kunkel, T., Roberts, J. D. & Zabour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
20. Standring, D. N., Ou, J. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9338–9342.
21. Chisari, F., Fillipi, P., MacLachlan, A., Milich, D., Riggs, M., Lee, S., Palmiter, R., Pinkert, C. & Brinster, R. (1986) *J. Virol.* **60,** 880–887.
22. Robinson, H. L. (1967) *Proc. Natl. Acad. Sci. USA* **57,** 1655–1662.
23. Fuller, S. D. (1987) *Cell* **48,** 923–934.
24. Summers, J., Smith, P. & Horwich, A. (1990) *J. Virol.* **64,** 2819–2824.
25. Kuroki, K., Floreani, M., Mimms, L. & Ganem, D. (1990) *Virology* **176,** 620–624.
26. Macrae, D., Bruss, V. & Ganem, D. (1991) *Virology*, in press.