JOURNAL OF VIROLOGY, Nov. 1995, p. 6652–6657 Vol. 69, No. 11
0022-538X/95/$04.00+0

Functions of the Internal Pre-S Domain of the Large Surface Protein in Hepatitis B Virus Particle Morphogenesis

VOLKER BRUSS* AND KERSTIN VIELUF

Department of Medical Microbiology, University of Göttingen, D-37075 Göttingen, Germany

Received 9 June 1995/Accepted 10 August 1995

The large hepatitis B virus (HBV) surface protein (L) forms two isomers which display their N-terminal pre-S domain at the internal and external side of the viral envelope, respectively. The external pre-S domain has been implicated in binding to a virus receptor. To investigate functions of the internal pre-S domain, a secretion signal sequence was fused to the N terminus of L (sigL), causing exclusive expression of external pre-S domains. A fusion construct with a nonfunctional signal (s25L), which corresponds in its primary sequence to sigL cleaved by signal peptidase, was used as a control. SigL was N glycosylated in transfected COS cells at both potential sites in pre-S in contrast to s25L or wild-type L, confirming the expected transmembrane topologies of sigL and s25L. Phenotypic characterization revealed the following points. (i) SigL lost the inhibitory effect of L or s25L on secretion of subviral hepatitis B surface antigen particles, suggesting that the retention signal mapped to the N terminus of L is recognized in the cytosol and not in the lumen of the endoplasmic reticulum. (ii) SigL was secreted into the culture medium even in the absence of the major HBV surface protein (S), while release of an L mutant lacking the retention signal was still dependent on S coexpression. (iii) s25L but not sigL could complement an L-negative HBV genome defective for virion secretion in cotransfections. This suggests that the cytosolic pre-S domain, like a matrix protein, is involved in the interaction of the viral envelope with preformed cytosolic nucleocapsids during virion assembly.

The hepatitis B virus (HBV) is an enveloped DNA virus with a unique replication strategy (for a review, see reference 13) which causes acute and chronic infections of the liver in humans. The virion has a diameter of 42 nm and consists of an icosahedral nucleocapsid and a detergent-sensitive envelope which contains three surface proteins. The virion is probably formed by interaction of a preformed cytosolic nucleocapsid with the surface proteins at a pre-Golgi membrane and secreted by the constitutive secretory pathway (12, 17). The three HBV surface proteins have several unusual features. (i) They are translated from a single open reading frame of the viral genome by the usage of three different start codons (Fig. 1A). Therefore, the 226-amino-acid sequence of the small surface protein (S) is repeated at the C termini of the middle (M) and large (L) surface proteins, which carry the additional N-terminal 55-amino-acid pre-S2 domain or the 176-amino-acid (subtype ad) pre-S2 plus pre-S1 domain, respectively (16). (ii) The surface proteins are not only incorporated into virion envelopes but are, in addition, secreted in large amounts from infected hepatocytes or transfected cells as spherical and filamentous subviral lipoprotein particles with a diameter of 22 nm, referred to as hepatitis B surface antigen (HBsAg) particles. The release of subviral particles (5, 6, 24, 28) or virions (2) is inhibited in a dose-dependent fashion by overexpression of the L protein. This effect might have relevance for the pathogenesis of hepatitis B (7). The biological significance of the retention is unknown. (iii) The HBV surface proteins gain a complex transmembrane topology upon translation at the endoplasmic reticulum (ER) and, most unusual, the topology of a fraction of the L proteins in the ER does not correspond to its topology in the viral envelope (3, 23, 27) (Fig. 1A).

The S protein has a type I signal at its N terminus and an internal type II signal which cause the protein to traverse the membrane at least twice and to form a cytosolic loop as well as a luminal domain (10) (Fig. 1A). The latter forms the main epitopes of the HBsAg and contains an N-glycosylation site which is, however, used in only approximately half of the peptide chains for unknown reasons. The C terminus of S is very hydrophobic and is believed to be embedded in the lipid bilayer. The M protein has a similar transmembrane topology and exposes the pre-S2 domain in the ER lumen (9) (Fig. 1A). The pre-S domains of the L protein are first located on the cytosolic side of the ER, and the protein spans the membrane with the type II signal and hydrophobic C terminus in its S domain (Fig. 1A). The transmembrane topology of the S and M proteins is, as expected, preserved during viral particle assembly. Therefore, the pre-S2 domain of M and the major HBsAg epitopes in the S domain appear on the surface of secreted viral particles. Unusually, however, the transmembrane topology of approximately half of the L chains changes posttranslationally in such a way that their pre-S domains are exposed on the surface of secreted virions (3). As a consequence, the particles contain two forms of the L protein which carry an external (e-pre-S) or internal (i-pre-S) pre-S domain, respectively. The mechanism of the posttranslational refolding process is unknown to date.

Infection experiments using the duck hepatitis B virus model implied that one important function of the e-pre-S domain is binding to a putative virus receptor (19). Other studies showed that the L protein of HBV is necessary for virion formation (2, 31), implying an additional function of L in virion morphogenesis. In this work we investigated the functions of the i-pre-S form of L in viral particle assembly by characterizing an L mutant which expressed exclusively e-pre-S domains. We found that the i-pre-S domains mediate the intracellular retention of subviral HBsAg particles and were necessary for virion formation. Our data support the hypothesis that the i-pre-S domains function as a matrix protein by mediating the contact between viral envelope and nucleocapsid during virion assem-

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Göttingen, Kreuzbergring 57, D-37075 Göttingen. Phone: 49 551 39 5759. Fax: 49 551 39 5860.

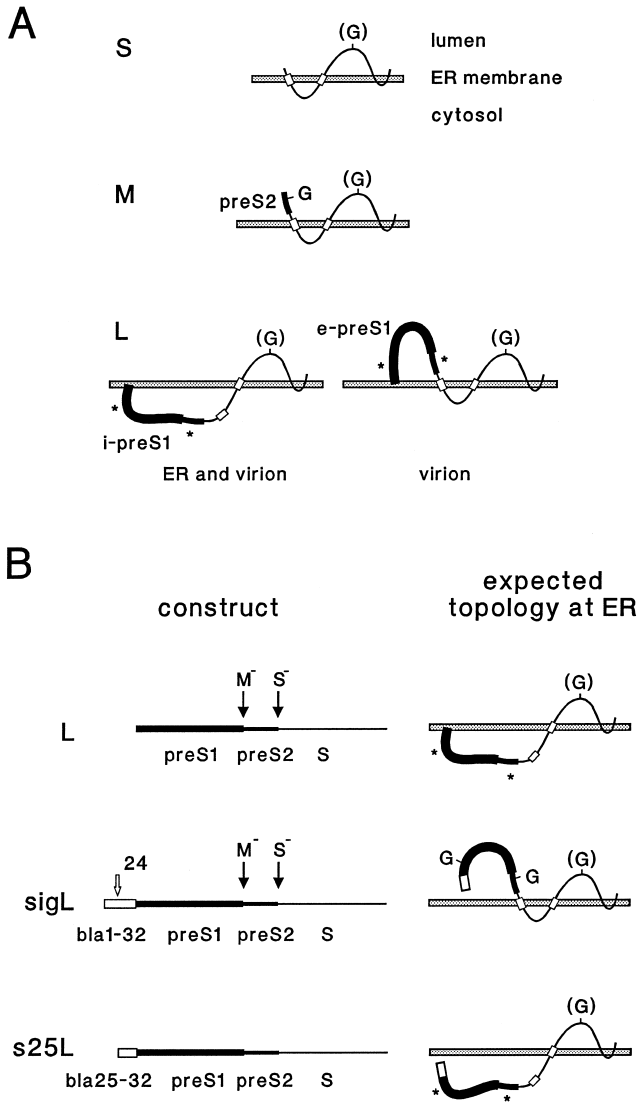

FIG. 1. Transmembrane topology of HBV surface proteins and fusion constructs. (A) The HBV surface proteins have a complex transmembrane topology in the ER membrane (stippled bar). The small HBV surface protein (S, small line) traverses the membrane with the N-terminal type I and the central type II signals (open boxes) and probably twice with its hydrophobic C-terminal region. The luminal loop is partially N glycosylated (G in parentheses). The medium-sized surface protein (M) has a similar topology and displays the additional N-glycosylated N-terminal pre-S2 domain (thicker line) in the ER lumen. The large surface protein (L) carries an additional N-terminal domain (pre-S1, thickest line). The pre-S1 and pre-S2 domains of L are first located at the cytosolic side of the ER membrane (i-pre-S1) and are not N glycosylated at the two potential sites (stars). In the virion, approximately half of the L proteins display their pre-S domains to the outside (e-pre-S1). (B) The sigL fusion construct carries the first 32 amino acids of β-lactamase (bla1–32), which contains a secretion signal (open box) fused to amino acid 7 of pre-S1. The signal causes translocation of pre-S1 and pre-S2 into the ER lumen and N glycosylation of the pre-S domains (G). The signal is cleaved between amino acids 23 and 24 (33) by signal peptidase. The s25L construct has a primary amino acid sequence identical to that of cleaved sigL except for the first 2 amino acids. The truncated signal in s25L (bla25–32) is not functional. The M^-S^- versions of L and sigL carry mutations of the start codons for M and S protein synthesis (arrows) to threonine codons.

bly. By generating a dual transmembrane topology, the pre-S domains of the L protein therefore fulfill two important functions in the viral life cycle on both sides of the viral envelope: binding to a virus receptor on the outside and to the nucleocapsid on the inside.

MATERIALS AND METHODS

Plasmid constructions. Simian virus 40 early promoter expression vectors for the HBV S protein (pSVBX24H) (14), for the simultaneous expression of L, M, and S proteins (pSV45H) (24), and for the expression of M protein without concomitant S expression (pSVM) (14) have been described previously. The plasmid $pRVL^-$ carries one and one-half head-to-tail copies of a replication-competent HBV DNA subtype adw2 (32) with an amber mutation in the pre-S1 region and has been described elsewhere (2). Plasmid $pSVLM^-S^-$ and $pSV45\text{-}81M^-S^-$ correspond to plasmids pSV45H and pSV45-81 (4), respectively, but carry mutations of both the M and S protein start codons to the threonine codon ACG. The plasmid pSVsigLa was constructed by PCR amplification of DNA coding for the 5′ 32 amino acids of the β-lactamase signal from plasmid pBluescript KS(+) (Stratagene) with upstream primer 5′CCTTGCAAAGCTTCCCT GATAAATGCTTC (primer 1 [the *Hin*dIII site is underlined]) annealing 5′ of the β-lactamase sequence and downstream primer 5′GCCTTTGCGAGG**TTT TACTTTCACCAGCG** (primer 2 [the β-lactamase sequence is in boldface type; the HBV sequence, nucleotide {nt} 2884 to 2873, is in lightface type; the numbering of the subtype adw2 is as follows: C of the unique *Eco*RI site on the plus strand of the cloned HBV genome is nt 1]). HBV DNA was PCR amplified with a primer complementary to primer 2 and a primer corresponding to HBV sequence nt 426 to 402 (primer 4). Both amplification products were mixed and spliced by a second PCR with primers 1 and 4, and the product was cut with *Hin*dIII and *Xba*I (at HBV nt 245) and inserted into *Hin*dIII-*Xba*I-cut simian virus 40 early promoter expression vector pSVM, resulting in plasmid pSVsigLa. The start codon of the β-lactamase signal in this construct was used only inefficiently (data not shown). Therefore, we changed the initiation context according to the rules of Kozak (20) by PCR with plasmid pSVsigLa as the start template and primers 5′CCTTGCAAAGCTTcc**ATG**gGTATTCAACATTTCC (the *Hin*dIII site is underlined; the start codon is in boldface type; introduced mutations are in lowercase letters) and primer 4. The mutations changed the second amino acid from serine to glycine. The *Hin*dIII-*Xba*I-cut PCR product was cloned into *Hin*dIII-*Xba*I-cut pSVM, resulting in plasmid pSVsigL. The region generated by PCR was sequenced to verify the expected sequence. The plasmid pSVs25L was constructed by cloning a *Hin*dIII-*Xba*I-cut PCR fragment generated from pSVsigL with upstream primer 5′CCTTGCAAAGCTTACC**AT G**GCAGAAACGCTGGTG (the *Hin*dIII site is underlined; the start codon followed by amino acid 25 of the β-lactamase sequence which was mutated from a proline to an alanine to allow strong initiation of protein synthesis is in boldface type) and primer 4 into *Hin*dIII-*Xba*I-cut pSVM. Sequencing confirmed the expected sequence.

Expression and characterization of viral surface proteins. Transient transfection of COS7 cells, metabolic pulse-chase labeling with [^{35}S]methionine, immunoprecipitation of HBV surface proteins from cell lysates and medium, endoglycosidase treatment, gel electrophoresis, and autoradiography have been described previously (4).

Assay for virion formation in transfected HepG2 cell cultures. Transient transfection of HepG2 cells, immunoprecipitation of viral particles from cell lysates and medium, the radioactive endogenous polymerase reaction, isolation of the viral genome, agarose gel electrophoresis, and autoradiography have been described previously (4).

RESULTS

Experimental approach. To investigate functions of the i-pre-S domain, we took a genetic approach and constructed and characterized a mutant L protein which carries only external e-pre-S domains. For this purpose, the first 32 amino acids of the bacterial β-lactamase, which contain a secretion signal, were fused to amino acid 7 of the pre-S sequence. The N-terminal signal in this construct (sigL) was expected to cause the cotranslational entry of the pre-S domain into the ER lumen (22) and to be cleaved between amino acid 23 (alanine) and 24 (histidine) by signal peptidase (Fig. 1B) (33). The N terminus of wild-type (WT) L (25) but not of sigL is myristylated. To distinguish whether new phenotypes of sigL are due to the alteration of the structure and primary amino acid sequence at the N terminus or are the consequence of the altered transmembrane topology we constructed a second mutant (s25L) which corresponds in its primary sequence to mature sigL cleaved by signal peptidase. This was achieved by fusing amino acids 25 to 32 of the β-lactamase signal preceded by a methionine residue instead of the histidine residue in cleaved sigL to the same site of pre-S. In addition, amino acid 25 was changed from a proline to a glycine to allow efficient initiation of protein translation. The truncated signal sequence of s25L is

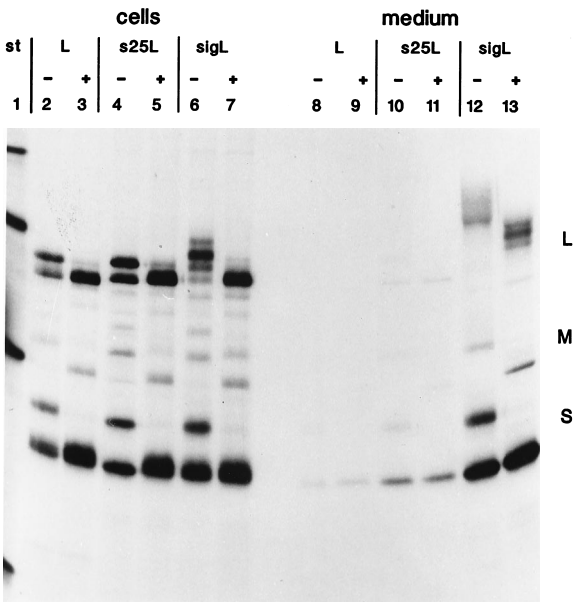

FIG. 2. Secretion and N glycosylation of WT L, s25L, and sigL proteins. Pulse-chase-labeled and immunoprecipitated proteins from medium and from Nonidet P-40 cell lysates were treated with endoglycosidase H in the case of cell-derived material and with endoglycosidase F in the case of medium-derived samples (+) or were mock treated (−). The WT L protein is partially N glycosylated at one site in the S domain (lanes 2 and 3) and inhibits the secretion of the S protein (lanes 8 and 9). The sigL protein forms four species (lane 6) carrying zero to three glycan residues (compare with lane 7), confirming the expected transmembrane topology. sigL lost the inhibitory effect on secretion (lane 12). Endoglycosidase F treatment reduced the smear formed by sigL (lane 13). The s25L control showed a WT phenotype (lanes 4, 5, 10, and 11). The M protein was expressed in small amounts; the S protein was expressed in larger amounts from the expression plasmids. (st, molecular mass standards [69, 46, 30, and 14.3 kDa]).

not functional. Therefore, s25L and cleaved sigL have an identical primary amino acid sequence except for the first 2 amino acids, but their pre-S domains have an opposite orientation directly after synthesis at the ER membrane.

The cytosolically orientated pre-S domain is required for intracellular HBsAg retention. The mutant L proteins were first characterized by transient expression in transfected COS7 cells. The proteins were visualized by metabolic pulse-chase labeling with [^{35}S]methionine, immunoprecipitation with an antiserum against HBsAg (anti-HBs) from cell lysates and culture medium, polyacrylamide gel electrophoresis, and autoradiography. The WT L protein is visible as a doublet with molecular masses of 39 and 42 kDa (Fig. 2, lane 2). The protein in the upper band carried one glycan residue as confirmed by endoglycosidase treatment (Fig. 2, lane 3) at the facultative N-glycosylation site in the S domain (16). The S protein and small amounts of the M protein were also expressed from the plasmid. They, too, appear as doublets of 24 and 27 kDa and 33 and 36 kDa, respectively, because of the facultative N glycosylation of the S domain. Release of the proteins into the culture medium was drastically reduced (compare Fig. 2, lanes 8 and 9, with Fig. 3, lanes 2 and 3) because of the inhibitory effect of the L protein, which was expressed in relatively large amounts in this experiment.

The sigL mutant formed four bands between 39 and 48 kDa (Fig. 2, lane 6). The upper three bands represent N-glycosylated versions carrying one to three glycans as apparent from the endoglycosidase treatment (Fig. 2, lane 7) and the approximately 3-kDa difference in molecular mass between adjacent bands. The sigL protein carries three potential N-glycosylation sites: at Asn-15 of the pre-S1 sequence, Asn-4 of pre-S2, and Asn-146 of the S domain. The triple-N-glycosylated sigL protein demonstrates that the two glycosylation sites in pre-S are potential substrates for N glycosylation and have been translocated into the ER lumen by the N-terminal secretion signal as expected (Fig. 1B). We assume that the less-glycosylated sigL proteins had the same transmembrane topology and were the result of partial usage of the N-glycosylation sites.

The sigL protein is efficiently released from the cells together with S protein (Fig. 2, lane 12) and formed a smear to higher molecular mass probably because of modification of the sugar moieties. The smear could be reduced by endoglycosidase F treatment (Fig. 2, lane 13) but not to a single band, for unknown reasons. The s25L construct was N glycosylated like WT L, indicating the expected cytosolic location of its pre-S domains (Fig. 2, lanes 4 and 5, and Fig. 1B) and caused intracellular retention of the surface proteins that was almost as efficient as that caused by WT L (Fig. 2, lane 10).

From the comparison of sigL and s25L we conclude that loss of the retention effect of sigL is most probably not caused by the alteration of the primary N-terminal amino acid sequence but is a consequence of the altered transmembrane topology. The retention signal has been mapped to the N terminus of L (21, 26). The experiment shows that this sequence has to be located on the cytosolic side of the ER in order to function and is ineffective on the luminal side.

SigL secretion is independent of S-protein coexpression. As mentioned above, the L protein inhibits the secretion of subviral particles (Fig. 2, lanes 2 and 8). Therefore, it was expected that L protein expressed in the absence of M and S proteins, which was achieved by mutation of the corresponding start codons to threonine codons (2), is not able to form subviral particles and to be released from cells. This was indeed found to be the case (Fig. 3, lanes 6 and 7). The M protein, on the other hand, which has no inhibitory effect on subviral HBsAg particle release, appeared in the culture medium when expressed in the absence of S (Fig. 3, lanes 4 and 5) although with reduced efficiency. The inhibitory effect of L on HBsAg secretion can be abolished, e.g., by deletion of the N-terminal 80 amino acids of pre-S1 (4) which contain the retention signal. The pre-S domains of this N-terminally truncated L mutant (45-81) are not cotranslationally translocated into the ER lumen, as evident from the absence of N glycosylation of the pre-S2 domain (4). The S and M protein start codons in 45-81 were altered to threonine codons (45-81M$^-$S$^-$) to allow expression of 45-81 without concomitant S and M synthesis. The construct 45-81M$^-$S$^-$ did not inhibit surface protein secretion when coexpressed with WT S protein, as expected (Fig. 3, lanes 12 and 13); however, its release into the culture medium was still dependent on concomitant S expression (Fig. 3, lanes 10 and 11). The sigLM$^-$S$^-$ mutant expressed without M and S proteins, on the other hand, was secreted (Fig. 3, lanes 8 and 9). We deduce from these observations that the L protein with cytosolic pre-S domains needs help from the S protein to form secreted subviral particles, whereas the L protein with luminal pre-S domains is independent of this helper function.

Virion formation depends on the cytosolic exposure of the pre-S domain. Studies on the morphogenesis of hepatitis B virions revealed that both the S and L proteins but not the M protein are necessary to secrete virions in transfected cells (2) and in vivo (11). We asked whether the L isomer with cytosolic pre-S domain is required to form virions. For this purpose, human hepatoma cells (HepG2) were transiently transfected with a cloned replication-competent HBV genome which carried a stop codon in the pre-S1 open reading frame (2). The

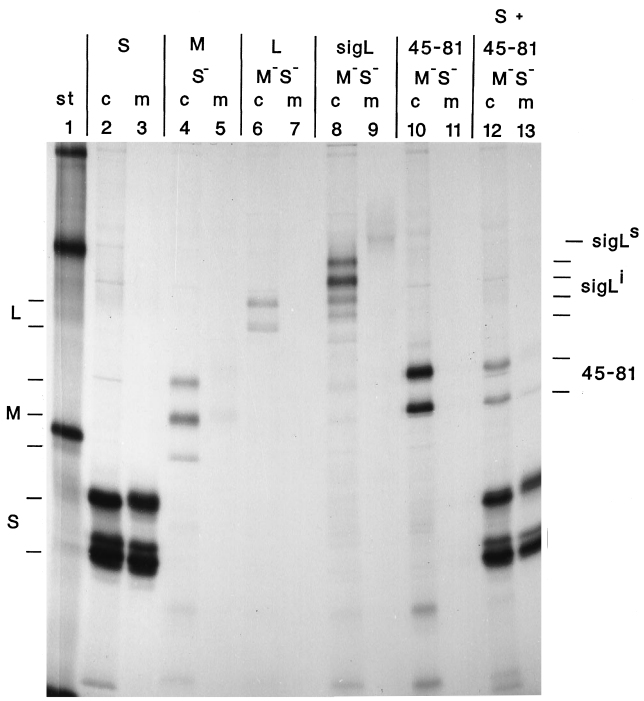

FIG. 3. Secretion of WT L, sigL, and N-terminally truncated L protein without concomitant S expression. Proteins were pulse-chase labeled and immunoprecipitated from Nonidet P-40 cell lysates (c) and medium (m). Approximately half of the S protein was secreted after a 1-h pulse and 24-h chase (lanes 2 and 3). The glycosylated versions of the M protein were secreted in the absence of S, although less efficiently (lanes 4 and 5). The L protein was not released in the absence of S and M (lanes 6 and 7) in contrast to the sigL (sigL^i, lane 8), which was secreted (sigL^s, lane 9). An N-terminally truncated L protein (45-81M$^-$S$^-$) lacking the retention signal was only secreted in the presence of S (lanes 10 to 13). (st, molecular mass standards, as in the legend to Fig. 2).

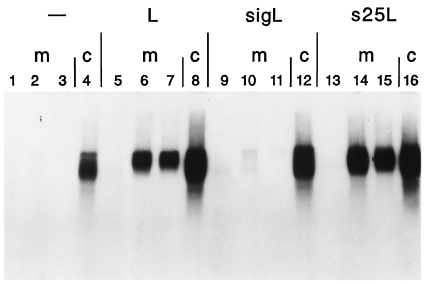

FIG. 4. Virion formation by transcomplementation of an L-negative HBV genome with WT L, s25L, and sigL. An L-negative HBV genome was transfected into HepG2 cells (—) or cotransfected with expression plasmids for WT L, sigL, and s25L proteins. Nucleocapsids were immunoprecipitated from cells (c) with anti-HBc. Virions were immunoprecipitated from the medium (m) with anti-HBc in the absence of detergent (left m lanes), with anti-HBc in the presence of detergent (central m lanes), and with anti-HBs (right m lanes). The viral genomes were labeled by a radioactive endogenous polymerase reaction and visualized by agarose gel electrophoresis. The L-negative genome produced intracellular nucleocapids (lane 4) which were not secreted as naked (lane 1) or enveloped (lanes 2 and 3) nucleocapsids. This defect could efficiently be complemented in *trans* by coexpression of WT L protein (lanes 6 and 7) or the s25L control (lanes 14 and 15) but not by sigL (lanes 10 and 11). A very small amount of virions is detectable in lane 10 (see Discussion).

cells expressed intracellular nucleocapsids which could be detected in cell lysates after immunoprecipitation with a capsid-specific antibody (anti-HBc) by a radioactive endogenous polymerase reaction (18). In this reaction, the viral DNA polymerase which is encapsidated in the nucleocapsids incorporates added labeled deoxynucleotides by elongation of the plus strand of the partial double-stranded viral genome. The labeled genomes were detected after isolation and separation on an agarose gel by autoradiography (Fig. 4, lane 4). The cells did not release virions into the culture medium, as apparent from immunoprecipitations with anti-HBs (Fig. 4, lane 3) or anti-HBc in the presence of detergent (Fig. 4, lane 2), nor did they release naked nucleocapsids which would be immunoprecipitated with anti-HBc in the absence of detergent (Fig. 4, lane 1). This defect of the L-negative HBV genome could be complemented in *trans* by cotransfection of an expression vector for the missing L protein (Fig. 4, lanes 5 to 8) (2). Virion formation could also be restored by the s25L construct (Fig. 4, lanes 13 to 16) but not by the sigL mutant (Fig. 4, lanes 9 to 12). This demonstrates that exposure of the pre-S domains of L on the cytosolic side of the ER is necessary for virion formation. The result is consistent with the proposed function of the L protein carrying the i-pre-S domain as a matrix protein (Fig. 5).

DISCUSSION

The HBV L protein has an unusual feature: the N-terminal cytosolic pre-S domains (i-pre-S) of some L chains traverse a cellular membrane or the viral envelope in a posttranslational manner such that the pre-S domains are subsequently located in the lumen compartment or on the external surface of virions (2, 23, 27) (e-pre-S, Fig. 5). Therefore, the viral envelope contains two isomers of L with i-pre-S and e-pre-S domains, respectively. Two views of the significance of this partial posttranslational folding process are possible. According to the first, the i-pre-S form is only an intermediate state during the maturation of the e-pre-S form which functions as an adapter for a virus receptor (19). The i-pre-S form in the viral envelope would then have no other separate function in the HBV live

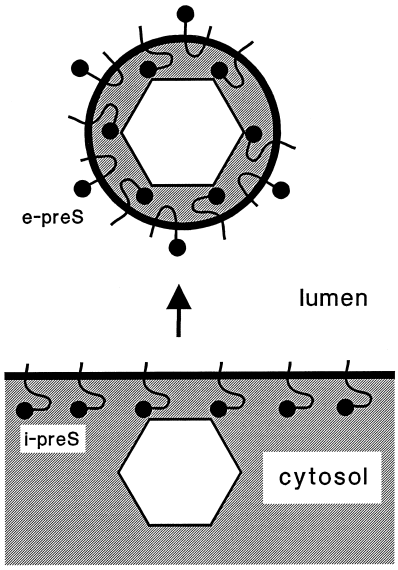

FIG. 5. Model for the matrix protein function for the i-pre-S form of the L protein. The pre-S domains of L are shown as filled circles. The topology of the S domain is simplified by leaving out the hydrophobic C-terminal region. The S and M proteins are omitted. The e-pre-S domain on the surface of secreted virions probably has the function to bind to a putative virus receptor. The presented data suggest that the i-pre-S domain of L functions as a matrix protein to contact the nucleocapsid during virion morphogenesis.

cycle and would be the result of incomplete maturation and passive incorporation. Or, according to the second, both forms have their own distinct functions. By generating two topologies a single protein could then serve two sets of functions.

The experiments presented in this paper strongly argue for the second alternative. Our approach was to characterize an L mutant (sigL) which generates exclusively the e-pre-S form. We tried to achieve this by fusion of a signal sequence to the N terminus of L. On the basis of the known effect of N-terminal secretion signals on protein topology and the known topogenic elements in the L protein (23), we anticipated that sigL has a topology like the e-pre-S form of WT L (Fig. 1B). This expectation was confirmed by the N-glycosylation pattern of the sigL protein (Fig. 2, lane 6). At least, the triple-N-glycosylated version of sigL exposes the pre-S domains in the ER lumen. The sigL chains with fewer glycan residues were probably due to incomplete N glycosylation like that known for the S domain and not the consequence of different transmembrane topologies. This view is supported by an internal pre-S1 deletion mutant of L which was cotranslationally translocated and partially N glycosylated like sigL (27). In this case, protease protection experiments confirmed that all four differently glycosylated forms of the L derivative had identical transmembrane topologies. A fusion construct similar to sigL was expressed in a cell-free in vitro system where the same N-glycosylation pattern was also found (23). The N glycosylation of pre-S in all these constructs demonstrates that this domain is a potential substrate for the N-glycosylating enzyme. The fact that these sites are not used in pre-S of WT L argues again for the model in which the WT pre-S domain traverses the membrane by a posttranslational refolding mechanism which does not involve the conventional translocation apparatus at the ER.

The sigL protein lost the inhibitory effect of WT L on HBsAg particle secretion (Fig. 2). The sequence of L which is responsible for the retention was mapped to its N terminus (21, 26). Since the s25L construct has virtually the same primary amino acid sequence as sigL and still contained the retention signal (Fig. 2, lanes 4 and 10), it is most likely that this signal is also present in sigL but was unable to function because it was located in a different cellular compartment. Apparently, this signal is recognized at the cytosolic side of the ER membrane by cytosolic factors. One candidate for such a factor is cytosolic Hsc70, which has been determined to bind to i-pre-S but not e-pre-S domains of the duck HBV L protein (30). Another possibility is a phosphorylating-dephosphorylating factor, because it was shown that the i-pre-S form of the duck HBV L protein is phosphorylated, in contrast to the e-pre-S form (15).

Deletion of the N-terminal retention signal of L led to efficient cosecretion of the deleted L protein together with M and S proteins (5, 6, 24, 28). It was, therefore, possible that such an L mutant gained the ability to form subviral HBsAg particles and to be secreted in the absence of S and M proteins. However, coexpression of S protein was still necessary (Fig. 3, lanes 11 and 13). Because secretion of sigL was independent of S coexpression (Fig. 3, lane 9), we infer that the cytosolic location of the pre-S domain was responsible for this effect. A globin-S fusion construct showed a similar behavior (1). This chimera displayed the globin domain on the cytosolic side of the ER and was retained within cells. Fusion of a signal sequence to its N terminus caused cotranslational translocation of the globin domain into the ER lumen and secretion without concomitant S expression. In this case, however, coexpression of the S protein could not mobilize the globin-S fusion lacking the N-terminal secretion signal, which demonstrates a specific interaction of the i-pre-S form of L with the S protein which was not possible with the globin-S fusion.

This points to a specific cooperation between the L and S proteins in particle morphogenesis. At first, the interaction of the S protein with L is required to override the N-terminal retention signal of L. In addition, an L protein with a cytosolic pre-S domain is unable to form subviral particles independent of the presence or absence of the retention signal. The S protein seems therefore to be necessary to promote the incorporation of the L protein into subviral particles or the virion envelope in a step which is independent of the retention function.

The sigL mutant was unable to complement an L-negative HBV genome defective for virion secretion (Fig. 4, lanes 10 and 11). Because the sigL protein could be secreted as a component of subviral particles from transfected COS cells (Fig. 2, lane 12), it is likely that the formation of virions rather than their secretion was affected. This deficiency was the consequence of the altered transmembrane topology, as evident from the comparison with s25L (Fig. 4, lanes 14 and 15). It is unlikely that the deficiency of sigL was due to general misfolding, because the protein was stable and was incorporated into subviral particles (Fig. 2, lane 12). Therefore, we conclude that the i-pre-S form of L was necessary for the formation of virions.

An attractive model is that the i-pre-S domain serves as an anchor to recruit nucleocapsids to budding sites. This model explains the heretofore not understood observation that both the S and the L proteins are necessary for virion formation (2). The L protein with i-pre-S domain and the S protein expose different structures at the cytosolic side of the cellular membrane where the interaction with the nucleocapsid occurs. It is conceivable that the i-pre-S domain mediates the contact to the nucleocapsid during virion morphogenesis in a way similar to that proposed for the cytosolic tail of the spike protein E2 for Semliki Forest virus (29). The C-terminal part of pre-S1 starting with residue 103 and the pre-S2 domain and the N-terminal sequence of the S domain of L up to signal II are candidates for this function, because these sequences are exposed on the cytosolic side of the ER membrane and an N-terminal truncation of L up to amino acid 102 was compatible with virion formation (4). Recently, Dyson and Murray (8) proposed a direct interaction of the pre-S domain of L with HBV nucleocapsids based on in vitro binding studies. Apparently, this function of the i-pre-S domains is conserved between the related woodchuck hepatitis virus and HBV because the woodchuck hepatitis virus L protein could partially restore the formation of virions by cotransfection with the L-negative HBV genome (14). This was not possible with the less-related duck HBV L protein (14).

The assay for virion formation by transcomplementation of the cotransfected L-negative HBV genome and sigL expression plasmid resulted in a faint signal for enveloped virions (Fig. 4, lane 10). This may indicate that the requirement for the i-pre-S domain in virion morphogenesis is not absolute. However, an alternative explanation is also conceivable: the β-lactamase signal was fused to codon 7 of pre-S1. Codon 12 of pre-S1 is a methionine codon which is homologous to the L protein start site in HBV subtype ay. Internal initiation of protein synthesis at this site would generate an L protein with a WT phenotype. Our initial sigL expression construct carried the original initiation codon of the β-lactamase signal (sigLa; see Materials and Methods), which is a weak one according to the rules of Kozak (20), and showed extensive initiation of protein synthesis at pre-S1 codon 12 (data not shown). We therefore changed the initiation codon of the β-lactamase se-

quence in the sigL expression plasmid to a strong one, according to the rules of Kozak (20), which seemed to abolish initiation at pre-S1 codon 12 (Fig. 2, lane 6). However, it is possible that small amounts of this N-terminally truncated L protein, which could not be detected in the protein analysis but which were sufficient for the observed limited virion production in the cotransfection experiment, were still expressed.

HBV developed an extreme economy in using the limited coding capacity of its small 3.2-kb genome: the viral genome has extensively overlapping reading frames and directs the expression of different proteins from single reading frames by using alternative start codons. Here, we described another example of this remarkable economy: by generating two different transmembrane topologies, the L protein of HBV is able to fulfill functions at the interior and exterior sides of the viral envelope—binding to the nucleocapsid and to the virus receptor.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft, grant BR 1018/2-2.

REFERENCES

1. **Bruss, V., and D. Ganem.** 1991. Mutational analysis of hepatitis B surface antigen particle assembly and secretion. J. Virol. **65:**3813–3820.
2. **Bruss, V., and D. Ganem.** 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Natl. Acad. Sci. USA **88:**1059–1063.
3. **Bruss, V., X. Lu, R. Thomssen, and W. H. Gerlich.** 1994. Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. EMBO J. **13:**2273–2279.
4. **Bruss, V., and R. Thomssen.** 1994. Mapping a region of the large envelope protein required for hepatitis B virion maturation. J. Virol. **68:**1643–1650.
5. **Cheng, K.-C., G. L. Smith, and B. Moss.** 1986. Hepatitis B virus large surface protein is not secreted but is immunogenic when selectively expressed by recombinant vaccinia virus. J. Virol. **60:**337–344.
6. **Chisari, F. V., P. Filippi, A. McLachlan, D. R. Milich, M. Riggs, S. Lee, R. D. Palmiter, C. A. Pinkert, and R. L. Brinster.** 1986. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. J. Virol. **60:**880–887.
7. **Chisari, F. V., K. Klopchin, T. Moriyama, C. Pasquinelli, H. A. Dunsford, S. Sell, C. A. Pinkert, R. L. Brinster, and R. D. Palmiter.** 1989. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell **59:**1145–1156.
8. **Dyson, M. R., and K. Murray.** 1995. Selection of peptide inhibitors of interaction involved in complex protein assemblies: association of the core and surface antigens of hepatitis B virus. Proc. Natl. Acad. Sci. USA **92:** 2194–2198.
9. **Eble, B. E., V. R. Lingappa, and D. Ganem.** 1990. The N-terminal (pre-S2) domain of a hepatitis B virus surface glycoprotein is translocated across membranes by downstream signal sequences. J. Virol. **64:**1414–1419.
10. **Eble, B. E., D. R. MacRae, V. R. Lingappa, and D. Ganem.** 1987. Multiple topogenic sequences determine the transmembrane orientation of hepatitis B surface antigen. Mol. Cell. Biol. **7:**3591–3601.
11. **Fernholz, D., P. R. Galle, M. Stemler, M. Brunetto, F. Bonino, and H. Will.** 1993. Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier. Virology **194:**137–148.
12. **Ganem, D.** 1991. Assembly of hepadnaviral virions and subviral particles. Curr. Top. Microbiol. Immunol. **168:**61–83.
13. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
14. **Gerhardt, E., and V. Bruss.** 1995. Phenotypic mixing of rodent but not avian hepadnavirus surface proteins into human hepatitis B virus particles. J. Virol. **69:**1201–1208.
15. **Grgacic, E. V. L., and D. A. Anderson.** 1994. The large surface protein of duck hepatitis B virus is phosphorylated in the pre-S domain. J. Virol. **68:**7344–7350.
16. **Heermann, K., U. Goldmann, W. Schwartz., T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-S sequence. J. Virol. **52:**396–402.
17. **Huovila, A. J., A. M. Eder, and S. D. Fuller.** 1992. Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J. Cell Biol. **118:**1305–1320.
18. **Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson.** 1973. DNA polymerase associated with human hepatitis B antigen. J. Virol. **12:**995–1005.
19. **Klingmüller, U., and H. Schaller.** 1993. Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor. J. Virol. **67:**7414–7422.
20. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eucaryotic ribosomes. Cell **44:**283–292.
21. **Kuroki, K., R. Russnak, and D. Ganem.** 1989. Novel N-terminal amino acid sequence required for retention of a hepatitis B virus glycoprotein in the endoplasmic reticulum. Mol. Cell. Biol. **9:**4459–4466.
22. **Lingappa, V. R., J. Chaidez, C. S. Yost, and J. Hedgpeth.** Determinants for protein localization: beta-lactamase signal sequence directs globin across microsomal membranes. Proc. Natl. Acad. Sci. USA **81:**456–460.
23. **Ostapchuck, P., P. Hearing, and D. Ganem.** 1994. A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis. EMBO J. **13:**1048–1057.
24. **Persing, D., H. Varmus, and D. Ganem.** 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science **234:**1388–1392.
25. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1987. The pre-S1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J. Virol. **61:**1672–1677.
26. **Prange, R., A. Clemen, and R. E. Streeck.** 1991. Myristylation is involved in intracellular retention of hepatitis B virus envelope proteins. J. Virol. **65:** 3919–3923.
27. **Prange, R., and R. E. Streeck.** 1995. Novel transmembrane topology of the hepatitis B virus envelope proteins. EMBO J. **14:**247–256.
28. **Standring, D. N., J. Ou, and W. J. Rutter.** 1986. Assembly of viral particles in Xenopus oocytes: pre-surface-antigens regulate secretion of the hepatitis B viral surface envelope particles. Proc. Natl. Acad. Sci. USA **83:**9338–9342.
29. **Suomalainen, M., P. Liljeström, and H. Garoff.** 1992. Spike protein-nucleocapsid interactions drive the budding of alphaviruses. J. Virol. **66:**4737–4747.
30. **Swameye, I., C. Kuhn, M. Hild, U. Klingmüller, H. Schaller.** Personal communication.
31. **Ueda, K., T. Tsurimoto, and K. Matsubara.** 1991. Three envelope proteins of hepatitis B virus: large S, middle S, and major S proteins needed for the formation of Dane particles. J. Virol. **65:**3521–3529.
32. **Valenzuela, P., M. Quiroga, J. Zaldivar, R. Gray, and W. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. UCLA Symp. Mol. Cell. Biol. **18:**57–70.
33. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184:**99–105.