The Pre-S2 Domain of the Hepatitis B Virus Is Dispensable for Infectivity but Serves a Spacer Function for L-Protein-Connected Virus Assembly

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The envelope of the human hepatitis B virus (HBV) contains three membrane proteins (L, M, and S). They accomplish different functions in HBV infectivity and nucleocapsid envelopment. Infectivity determinants have been assigned to the N-terminal part of the pre-S1 domain of the L protein and the antigenic loop of the S domain in the L and/or S protein. Nucleocapsid envelopment requires a C-terminal sequence within pre-S1, including the five N-terminal amino acids of pre-S2 as part of the L protein. However, the role of the M protein and the pre-S2 domain of the L protein are not entirely understood. We addressed this question and analyzed assembly competence and infectivity of viruses that lack the M protein and, at the same time, carry alterations in the pre-S2 domain of L. These include deletions, in part frameshift mutations and a randomization of virtually the entire pre-S2 sequence. We found that the M protein is dispensable for HBV in vitro infectivity. Viruses that lack the M protein and contain a mostly randomized pre-S2 sequence assemble properly and are infectious in HepaRG cells and primary human hepatocytes. While deletions of 20 amino acids in the pre-S2 domain of L protein allowed the production of infectious virions, more extended deletions interfered with assembly. This indicates that the pre-S2 domain of the L protein serves an important role for virus assembly, presumably as a spacer that supports conformational changes of L protein but does not participate as part of the M protein or as a subdomain of the L protein in virus entry.

The human hepatitis B virus (HBV) is the prototypic member of the family Hepadnaviridae. These enveloped DNA viruses infect primates, rodents, and birds. They show narrow host ranges and possess pronounced tropisms for hepatocytes where they replicate their genomes via reverse transcription (34, 39). Infected hepatocytes produce virions with a diameter of 42 nm and, in addition, noninfectious subviral particles (SVP) of spherical and filamentous morphology (7). The nucleocapsid of the virions is surrounded by a lipid membrane that contains the envelope proteins termed L (large), M (middle), and S (small). The S protein consists of 226 amino acids and includes four putative transmembrane domains. It drives particle budding and constitutes the major component of the envelope of SVP and virions. The M protein is composed of the S domain and the 55 hydrophilic N-terminal amino acids termed pre-S2. The L protein is further extended N terminally by the pre-S1 domain that consists of 108 or 119 genotype-dependent amino acids, respectively (Fig. 1A). Remarkably, the N-terminal pre-S1 domain of the L protein does not contain a signal sequence which could promote signal recognition particle-mediated endoplasmic reticulum insertion. Instead, it provides a recognition motif for the cellular N-myristoyltransferase. Accordingly, the pre-S1 domain becomes myristoylated early after or even during synthesis (5, 14, 28). At some later stage during particle assembly and matura-
FIG. 1. Expression, assembly, and infectivity of M-protein-deficient HBVs. (A) Schematic illustration of the genomic (HBV, HBV L−, HBV L− M−, and HBV L− M− S−) and the subgenomic plasmids (L and L109T) used for the production of M-protein-deficient HBV. Start codons of the viral proteins (C, core protein; P, polymerase; L, L protein; M, M protein; S, S protein; X, X protein) are marked by arrows. The pre-S1, pre-S2, and S domains of the L protein are highlighted in red, blue, and orange, respectively. The positions of point mutations introduced to abolish initiation of translation of L, M, and S proteins are depicted by a cross. (B) Analyses of secreted HBsAg (ELISA of SN from days 3 to 5) and intracellular L protein (Western blot at day 2 with monoclonal antibody MA/18-7) posttransfection of HuH-7 cells, with the plasmids indicated below. Note that despite the fact that endogenous promoter-driven expression of L protein (HBV) is significantly lower than ectopic coexpression controlled by the CMV promoter (L), HBsAg secretion remains similar. (C) HBV-specific DNA dot blot of fractions (indicated as 2 to 9) of an analytical CsCl density gradient centrifugation obtained from concentrated supernatants of transfected HuH-7 cells. Note that transfection with the HBV genomic construct (HBV) and the L-transcomplemented constructs expressing S protein results in secretion of both viral particles (VP, fractions 6 to 8) and free nucleocapsids (FC, fractions 3 to 4). Constructs that lack S-protein expression only form free nucleocapsids. (D) Infection of HepaRG cells with comparable amounts of concentrated cell culture supernatants of HuH-7 cells after transfection and/or cotransfection with HBV, HBV L− M−, HBV L− M− S− L, HBV L− M− S− L, and HBV L− M− S− + L. HBsAg secreted from days 7 to 11 postinfection was quantified by ELISA (black bars). A control infection was performed in the presence of 100 nM HBVpre-S/2-48myr, an HBV pre-S1-derived lipopeptide that blocks the pre-S1 receptor-mediated entry pathway of HBV (white bars). (E) HBcAg-specific immunofluorescence (green) and nucleus-specific DAPI staining (blue) of HepaRG cell 13 days postinfection with the concentrated cell culture supernatants of transfected HuH-7 cells indicated in the picture. Quantification of the number of HBcAg-expressing HepaRG cells (bar chart below) was done by single cell counting. The number of HBcAg-positive cells correlates to the secreted HBsAg antigen depicted in panel D.
demonstrated in well-defined antigenic loop of the S domain for virus entry have been fide bridges promote HDV and HBV entry is unresolved.

Using hepatitis delta virus (HDV), a surrogate system for studying HBV entry into host cells, the results for the requirements of the pre-S1 domain of the L protein were confirmed, indicating the high similarity of the entry pathways of both viruses (1). In this system, the third important infectivity determinant has been assigned to the antigenic loop of the S domain as a part of the M protein. The pre-S2 sequence PLSSIFSRIGDP, a constitutive subdomain of the L protein gave rise to some controversial discussions. Previously, LeSeyec et al. showed that consecutive deletions of 10 amino acids covering the part of the pre-S2 domain in the L protein (amino acids 114 to 163) that is not involved in nucleocapsid envelopment did not interfere with HBV infectivity (20). However, the constructs that were used to produce the recombinant mutants were not principally incapable of expressing M protein. Thus, the pre-S2 domain as a part of the M protein could still contribute to virus entry. Since the HBV pre-S2 sequence PLSSIFSRIGDP (called the translocation motif [TLM]), when artificially fused to proteins, is able to transport them across plasma membranes (25), Stoeckl et al. hypothesized that this motif may also be involved in viral particle translocation after receptor binding, endocytosis, and TLM activation. Experimental support came from their observation that HBV treated with HepG2 cell-derived endosomal extracts became "infectious" for the otherwise-nonpermissive HuH-7 cell line (35). Three independent subsequent investigations studied the role of the pre-S2 TLM in HBV and HDV entry in susceptible PHH and HepaRG cells. These studies concordantly showed that the TLM of pre-S2 has minor effects on HBV assembly and is dispensable for the infectivity of HBV and HDV (1, 16, 22).

In the present study we established a system that permits the production of HBV particles which lack the M protein and simultaneously carry deletions, frameshift mutations, and sequence permutations in the pre-S2 domain of the L protein. Using PHH, HepaRG cells, and a specific inhibitor of the pre-S-dependent HBV entry into hepatocytes, we show that (i) HBV lacking the whole M protein is infectious in vitro, (ii) HBV carrying a L protein with a mostly randomized pre-S2 amino acid sequence is infectious in the absence of the M protein, and (iii) deletions of pre-S2 sequences exceeding 20 amino acids interfere with virus assembly in a chain-length dependent manner, suggesting a spacer function in the L protein for proper nucleocapsid envelopment.

MATERIALS AND METHODS

Molecular cloning procedures. Plasmid P26 (HBV) harbors a 1.1-fold over length HBV genome (genotype D) in the backbone of the pcDNA3.1/Zeo(+) vector (Invitrogen). It was subcloned through insertion of the 3,381-bp SacI fragment of pCHT-9/3091 (24) into the equivalent restriction site of pcDNA3.1/ Zeo(–). Plasmid P27 (HBV L+) was derived from P26 through introduction of a point mutation at the start codon of the L open reading frame (ORF) to prevent L-protein expression (ATG to ACG). The two plasmids P33 (HBV L+ M-) and P35 (HBV L+ M-S) both contain a point mutation at the start codon of the M ORF (ATG to ACG) to prevent M-protein expression. P35 carries in addition a stop codon at position 6 of the S ORF (TCA to TAA) to abolish S protein expression. All mutations left the amino acid sequence unchanged.

Plasmid P21 (L) was used for cytomegalovirus (CMV)-promoter driven expression of the viral L protein. It was obtained through insertion of the PCR-generated ORF into NheI-HindIII sites of the pcDNA3.1/Zeo(+) vector. The derived plasmid P37 (L++) carries a point mutation at position Met-109 (GAC to ACA) of the L ORF to include a possible in-frame deletion mutations Δ114-133, Δ114-143, Δ114-153, Δ114-163, Δ144-163, Δ149-160, and Δ164-183 were constructed by SOEing PCR as previously described (20). This method is based on PCR amplification of two overlapping fragments located upstream and downstream of the deletion. For the first fragment, the primers S61 and ASmut were used. The second fragment encoding the sequence downstream of the mutations was generated by using the primers Smut and AS96 (Table 1). After extraction of the fragments from agarose gels, the two PCR products were mixed and subjected to a second round of amplification by using the primers S61 and AS96. Amplification products of the expected size were purified, and ligated into the P21 vector via EcoRI and XbaI restriction sites. The polymerase shift mutants S114-143, S114-163, and S114-183 were generated in a similar manner except that the primer S61 was replaced by S116, which led to the insertion of an adenine resulting in a frameshift into the pol-ORF. To scramble the codons 114 to 163 in the pre-S2 domain of the L protein (114-163transpos) the first fragment was generated by annealing and elongation of the primers S273 and AS274. The second fragment was generated by annealing and elongation of the primers S275 and AS277. After purification via agarose gel electrophoresis, the two products were mixed and underwent a second round of amplification using the primers S276 and AS277 (Table 1). After restriction with EcoRI and XbaI the amplified fragments were ligated into P21. The correctness of all sequences generated by PCR was verified by DNA sequencing.

Transfection of hepatoma cells. To produce the recombinant HBV particles, the human hepatoblastoma cell line HuH-7 (23) was transfected with appropriate mixtures of expression plasmids by using the polyethylenimine procedure (2). Shortly, 4 × 106 cells in a 10-cm dish were incubated for 6 h with the mixture of 75 μg of polyethylenimine and 10 or 12 μg of plasmid DNA. The cells were refreshed and further cultured for 8 days with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

Analysis of viral protein expression. To quantify S-protein expression and secretion, the supernatants of the transfected HuH-7 cells were collected between days 3 and 5 and tested for the presence of hepatitis B surface antigen (HBsAg) by the AsSYM (Abbott Laboratories). To analyze intracellular L-protein expression, HuH-7 cells at day 2 after transfection were washed once with phosphate-buffered saline (PBS), lysed in 25 mM Tris-HCl (pH 7.4)–250 mM NaCl–5 mM EDTA–1% Nonidet P-40, and then centrifuged at 12,000 × g for 10 min at 4°C to remove nuclei. The samples were then subjected to SDS-PAGE under reducing conditions. For subsequent Western blot analysis, we used the monoclonal antibody Ma18/7 (kindly provided by W. H. Gerlich, Giessen, Germany) recognizing the DPAF motif in the pre-S1 domain as the primary antibody and Alexa Fluor 680-conjugated goat anti-mouse IgG (Invitrogen/Molecular Probes) as the secondary antibody. L protein was visualized by using an Odyssey infrared imaging system (LI-COR).

Production, purification, and quantification of HBV particles. HBV particles with mutations in the viral surface proteins were obtained by cotransfection of HuH-7 cells with a 5:1 mixture of a 1.1-fold over-length HBV plasmid (HBV L-, HBV L+ M- or HBV L+ M-S) and a helper construct providing the respective L-protein mutants. Medium was collected between days 3 and 8. Virions were precipitated overnight at 4°C with 6% polyethylene glycol 8000 (PEG 8000; Sigma) and concentrated by centrifugation (12,000 × g, 60 min, 4°C). After suspension in 1/100 of the volume of PBS–10% fetal calf serum (FCS), the
TABLE 1. Oligonucleotides used for mutagenesis

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Mutant</th>
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<tr>
<td>S61</td>
<td>GTAGGCGGTACGGTGAGGTT</td>
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</tr>
<tr>
<td>AS96</td>
<td>TGGCGTGGCAACTGAAGGACA</td>
<td></td>
</tr>
<tr>
<td>S116</td>
<td>ATGCAATTTGAAATCCACCAATAACCTTCCACAA</td>
<td></td>
</tr>
<tr>
<td>ASmut</td>
<td>TGAACATGCGGAGATTCCTCACTGATGGCCTGAA</td>
<td>Δ114/133</td>
</tr>
<tr>
<td>Smut</td>
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<td>Δ114/143</td>
</tr>
<tr>
<td>ASmut</td>
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<td>Δ114/143</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Smut</td>
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<td>Δ114/153</td>
</tr>
<tr>
<td>ASmut</td>
<td>GATGTTCTCTACCAGAATTCCTCACGTGAGTTGGCCTGAA</td>
<td></td>
</tr>
<tr>
<td>Smut</td>
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<td>Δ114/163</td>
</tr>
<tr>
<td>ASmut</td>
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<td></td>
</tr>
<tr>
<td>Smut</td>
<td>CCTGGCTGAAATTTGGAAATGAACTTGAATGG</td>
<td>Δ114/163</td>
</tr>
<tr>
<td>ASmut</td>
<td>S273 TTGAGAAGTCCACCACGA</td>
<td>114-163 scrambled</td>
</tr>
<tr>
<td>Smut</td>
<td>AS276 ATCCTCAGGCCATGCAGTGG</td>
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<td>S274 CTAGGAATTTCTGATGTTGAGAATGAACTTGAATGG</td>
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<tr>
<td>ASmut</td>
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<td>Smut</td>
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<tr>
<td>ASmut</td>
<td>S276 ATCCTCAGGCCATGCAGTGG</td>
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<tr>
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<tr>
<td>ASmut</td>
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<td>Smut</td>
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RESULTS

HBV, lacking the M protein is infectious in vitro. The ORF for the three HBV envelope proteins overlaps with the coding region of the HBV polymerase. Therefore, in many cases, introduction of mutations into the envelope protein reading frame in “cis” also leads to amino acid exchanges in the viral polymerase. To overcome this limitation for our pre-S2 mutational studies and to ensure that the mutants we investigate are not affected by functional alterations of the polymerase, we followed an approach that relies on transcomplementation of two types of plasmids: one containing a replication-competent 1.1-fold HBV genome with point mutations that prevent the expression of L, M, or S protein (Fig. 1A) and another one encoding the L protein, L-protein mutants, or an L protein that lacks the preS2 start methionine (L<sub>M109T</sub>). To evaluate the suitability of this system we transfected HuH-7 cells with plasmids containing a replication competent 1.1-fold virus genome (designated HBV), a derivative with a mutation (ATG to ACG) in the pre-S start codon (designated...
HBV L−), a derivative carrying mutations (ATG to ACG) in the two start codons of pre-S1 and pre-S2 (designated HBV L−M−), or a derivative of L−M− with an artificially introduced stop codon (TCA to TAA) at position 6 of the S ORF (designated HBV L−M−S−). In none of the constructs did introduction of the respective mutations result in an amino acid change of the overlapping polymerase (Fig. 1A). Cell culture supernatants from days 3 to 5 posttransfection were analyzed for HBsAg levels by enzyme-linked immunosorbent assay (ELISA), which is indicative for all types of HBV particles including SVP. Cellular L protein (p39/gp42) was detected by Western blotting with the pre-S1 specific monoclonal antibody Ma18/7 (Fig. 1B). Transfection of HuH-7 cells with the wild-type HBV-plasmid resulted in secretion of HBsAg and cellular expression of both forms of the L protein (Fig. 1B, lane 2). As expected, the HBV L− and the HBV L−M− constructs promoted HBsAg secretion but were devoid of L-protein expression (Fig. 1B, lanes 3 and 4). The 1.1-fold HBV plasmid devoid of the expression of all three surface proteins did not support HBsAg secretion or L-protein expression (Fig. 1B, lane 5). Complementation of HBV L− and HBV L−M− with the L-protein expression vector (Fig. 1B, lane 6) rescued L-protein expression in the cells but had little influence on HBsAg secretion (Fig. 1B, lanes 6 and 7). This indicates that the cellular levels of transcomplemented L protein, although significantly increased compared to the wild-type HBV plasmid, are not sufficient to suppress HBsAg particle formation under the chosen conditions. Finally, cotransfection of HBV L−M−S− with the L-encoding plasmid led to L-protein expression in the cells but did not result in detectable HBsAg secretion between days 3 and 5 posttransfection (Fig. 1B, lane 8). This indicates that the promoter of S protein in the L-protein expression plasmid is silent.

To analyze and quantify virion production by the transfected HuH-7 cells, we performed analytical CsCl density gradient centrifugations with the cell culture supernatants and subjected the different fractions of the gradient to a HBV-specific DNA-Dot Blot (Fig. 1C). HuH-7 cells transfected with the 1.1-fold HBV plasmid produced virions (VP) with titers up to 9 × 107 genomes per ml of cell culture supernatant (row 1, fractions 6 to 8). A second peak accounting for ca. 30% of the total DNA banded at higher density and represented nonenveloped nucleocapsid (FC). Previous studies have shown that these particles are actively secreted by a pathway that differs from virion and SVP export (40). The role of these “naked DNA-containing capsids” in HBV replication is currently unclear. As expected, virion secretion was abolished in HuH-7 differentiated HepaRG cells in the absence and in the presence of a 100 nM concentration of the HBV pre-S1-derived peptide inhibitor HBVpre-S2-48myr. This inhibitor allows us to distinguish between authentic entry of virions and a possible envelope-protein-independent transfer of viral DNA, e.g., through artificial delivery of naked nucleocapsids. Infection was monitored by measuring HBsAg secretion and by quantification of the numbers of HbcAg-positive cells following immunofluorescence at day 13 postinfection. As expected, virions arising from the wild-type HBV construct were infectious, as shown by HBsAg secretion (Fig. 1D) and HbcAg-specific immunofluorescence staining of differentiated HepaRG cells (Fig. 1E). The infection could be efficiently competed by a 100 nM concentration of the inhibitory peptide HBVpre-S2-48myr. All supernatants derived from transfected HuH-7 cells that lack L- and/or S-protein expression (HBV L−M−, HBV L−M−S−, and HBV L−M−S− + L) were not infectious. However, the two preparations that lack M-protein expression (HBV L−M− + L and HBV L−M− + L M109T) showed comparable infectivity and comparable sensitivity to the inhibitor with the HBV wild type. These results demonstrate that the M protein of HBV is dispensable for both HBV assembly and HBV infectivity in vitro.

Analysis of assembly competence of M-protein-deficient HBV mutants carrying deletions and frameshift mutations in the pre-S2 domain of the L protein. Based on the observation that the M protein is dispensable for the infectivity of HBV in HepaRG cells, we next investigated the role of the pre-S2 domain as a constitutive part of the L protein in virion assembly. To that aim, we constructed a set of L-protein expression plasmids bearing incremental deletions that cover the whole pre-S2 domain except for the N-terminal five amino acids. In order to maintain the length of the pre-S2 construct and the respective L-expression vectors bearing the pre-S2 mutations, we monitored cel-
lular L-protein expression by Western blotting (Fig. 2B), HBsAg secretion by ELISA (not shown), and particle assembly by CsCl density gradient centrifugation of the cell culture supernatants and subsequent DNA dot blotting (Fig. 2C). As depicted in Fig. 2B (left) the L-protein deletions Δ144-163, Δ149-160, and Δ164-183 were expressed in similar levels compared to the full-length L protein. A slightly reduced expression was observed for the two deletion mutants affecting the TLM (Δ144-163 and Δ149-160) but not for the one affecting TM-1/sig-1 (Δ164-183) (right). With respect to the three pre-S2/polymerase frameshift mutations (S114-163 and S114-183) we could only verify protein expression for the shortest substitution (S114-143). Longer polymerase sequences replacing the pre-S2 sequence resulted in drastically reduced protein levels, indicating protein instability. In addition to the expected changes in the molecular weight, all mutant L proteins showed the typical double-band pattern arising from partial glycosylation in their S domains. This hints to an authentic topological orientation.

Figure 2C shows the DNA dot blots of fractions of the analytical CsCl density gradients from cell culture supernatants of transfected HuH-7 cells. Although all supernatants contained comparable amounts of nonenveloped nucleocapsids (FC), virion formation was detectable only for the full-length L protein (L), the four pre-S2 deletions (Δ114-133, Δ144-163, Δ149-160, and Δ164-183), and the frameshift mutation S114-143. L proteins with pre-S2 deletions (Δ114-143, Δ114-153, and Δ114-163) did not support virion formation. Virion assembly was also abolished when the two frameshift mutations S114-163 and S114-183 were examined. However, since these two L mutants are barely expressed, the deficiency in virion formation results from the lack of the envelope protein. Our observation that the L-deletion mutant Δ114-143 was incapable of virion formation, whereas the respective frameshift mutation S114-143 supported virion formation, indicates a sequence-independent function of these 30 pre-S2 amino acids. One such function could be the maintenance of a minimal interspace between the transmembrane domain at the beginning of the S protein and the nucleocapsid binding site. This hypothesis is consistent with the observation that mutants with increasing deletions (Δ114-153 and Δ114-163) were also unable to form virions.

HBV with deletions throughout the pre-S2 domain are infectious in vitro. To assess the infectivity of the M-protein-deficient pre-S2 deletion mutants, we infected differentiated HepaRG cells with the concentrated virus from the HuH-7
supernatants. At 12 days postinfection, we analyzed HBcAg expression in single cells using the core specific antiserum H363 (Fig. 3B and C). In addition, we quantified the secreted HBsAg between days 7 to 11 (Fig. 3A). To ensure that the authentic entry pathway was used, we performed all infections also in the presence of a 100 nM concentration of the pre-S1-derived lipopeptide HBVpre-S/2-48myr, which blocks a hepatocyte-specific HBV receptor. As shown in Fig. 3A, all assembly-competent pre-S2 deletion mutants (Δ114-133, Δ144-163, and Δ149-160) showed HBsAg secretion in the range of 30- to 45-fold above the cutoff for the ELISA. In particular, the M-protein-deficient viruses lacking the TLM (Δ149-160) or even a more extended sequence, including the TLM (Δ144-163), were fully infectious. In all cases, infection with the mutant viruses was sensitive to the myristoylated pre-S1-derived lipopeptide. This indicates that all viruses utilize the authentic pre-S1-mediated entry pathway. Consistent with the hypothesis that TM-1/sig-1 participates in membrane fusion, the deletion of TM-1/sig-1 (Δ164-183) resulted in a complete abrogation of the infectivity of the virus. This is consistent with recent findings of Lepere et al. (22).

M-protein-deficient HBV with a randomized pre-S2 sequence in its L protein properly assembles and is infectious in HepaRG cells and PHH. The results of our deletion and frameshift analyses already suggested that the pre-S2 domain in the HBV L protein is dispensable for infection but might serve a spacer function for virion assembly. To finally prove this hypothesis, we genetically randomized the coding region of the entire pre-S2 domain with the exception of the N-terminal 5 amino acids necessary for nucleocapsid interaction. The scrambled sequence enclosing amino acids 114 to 163 maintained the net amino acid composition and the codon usage of the wild-type preS2 (Fig. 4A). After cotransfection of HuH-7 cells with the respective plasmid pre-S2/114-163scrambled and the HBV L M- genomic construct, the mutant L protein was analyzed with regard to its expression level (Fig. 4B) and its competence to support virion envelopment and secretion (Fig. 4C). Although the intracellular expression level of the pre-S2-scrambled L protein was considerably lower compared to the wild-type L-protein, the glycosylation patterns of both proteins were identical (p39 and gp42). Analysis of the supernatants of the transfected HuH-7 cells revealed that despite the differences in intracellular L-protein levels, virion production remained unaffected. Since the lower concentration of the virus transcomplemented with the mutant L coincided with a similar reduction of the signal for naked capsids, it could be solely attributed to a variation of the transfection efficiency. Thus, scrambling of amino acids 114 to 163 in the pre-S2 domain does not substantially interfere with nucleocapsid envelopment and virion secretion.

The infectivity of the transcomplemented viruses was evaluated on HepaRG cells and PHH by measuring HBsAg secretion, HBeAg secretion, and cellular HBcAg expression. In parallel control experiments, we competed the infections with HBVpre-S/2-48myr to ensure that virus entry followed the authentic HBV-preS1-receptor mediated entry pathway. As shown in Fig. 4D, HepaRG cells infected with the HBV particles that were transcomplemented with the pre-S2-scrambled L released HBsAg between days 7 and 11 postinfection. HBsAg production was prevented by the myristoylated preS1-domains of HBV 3885

![FIG. 3. Infectivity of M-protein-deficient HBV with mutations in the pre-S2 domain of the L protein.](http://jvi.asm.org)
FIG. 4. Analysis of assembly and infectivity of an HBV mutant containing a scramble pre-S2 sequence. (A) Sequence of the HBV pre-S2 domain (genotype D) as part of the L protein (top) and the scrambled pre-S2 sequence encompassing amino acids 114 to 163 (below). Note that the N-terminal five amino acids remained unchanged in order to sustain nucleocapsid envelopment. The algorithm for scrambling amino acids 114 to 117 (YINI) differed from the one used to randomize amino acids 118 to 163. (B) HBV-pre-S1-specific Western blot (monoclonal antibody MA/18-7) of cellular extracts of HuH-7 cells, transfected with HBV L (L) and HBV L114-163scramble (114-163 scrambled). (C) HBV-DNA specific dot blot of fractions from analytical CsCl density gradients of the concentrated supernatants of HuH-7 cells after cotransfections with HBV L M− + L and HBV L M− + L114-163scrambled. Fractions containing nonenveloped nucleocapsids are labeled FC; fractions containing virions are labeled VP. Note that although the expression the pre-S2-scrambled L-protein was lower (Fig. 4B), virion formation was virtually unaffected. (D) Infection of HepaRG cells using comparable amounts of concentrated virus preparation obtained from HuH-7 cells, cotransfected with HBV L M− + L and HBV L M− + L114-163scrambled. HBsAg (above) secreted between days 7 and 11 postinfection was quantified by ELISA (black bars). Quantification on the single cell level was performed by counting HBeAg-positive cells 12 days p.i. (below). Control infections were performed in the presence of 500 nM HBVpre-S/2-48myr (white bars above). (E) Infection
peptide. In addition, we quantified infection events by immuno-fluorescence against the HBV core protein. The number of HBcAg-positive cells closely correlated with the HBsAg secretion (Fig. 4D, lower panel). On PHH, we monitored HBsAg and HBeAg secretion between days 4 and 6 and between days 7 and 9 (Fig. 4E) and finally visualized HBcAg expression at day 9 postinfection on the single cell level (Fig. 4F). The increase of both secretion markers in the culture supernatant between the two collection dates demonstrates a sustained genome replication similarly for both virus preparations. Again, the authenticity of the entry pathway was confirmed by the complete inhibition when infection took place in the presence of HBVpre-S/2-48myr (Fig. 4E and F). In all assays, the marker levels reached by the scrambled mutant were ~50% lower than that of the virus with wild-type L protein. Since this accurately reflects the different virion titers of the inocula used for infection (Fig. 4C), the specific infectivity of the particles that carry the pre-S2-scrambled L protein is identical compared to wild-type HBV.

**DISCUSSION**

The pre-S2 domain is a constitutive part of both the middle and the large surface protein of HBV. Previous investigations about the role of pre-S2 for HBV assembly and infectivity focused either on M or L protein but could not firmly rule out cross-compensatory effects of the respective remaining envelope protein. We provide here the first systematic approach in which the pre-S2 domain was mutated in the L-protein context in the complete absence of M protein. The present study substantiates the previous finding of Fernholz et al. that the M protein is not mandatory for HBV assembly and infectivity in vitro (9, 10). Beyond that, we show that the pre-S2 domain as such does not fulfill any specific sequence-dependent function for HBV infection despite the participation of its N-terminal five amino acids in capsid binding during morphogenesis. The whole 50 amino acids downstream of this short stretch can be completely randomized without major effects on virion assembly and infectivity. Since deletion of the N-terminal five pre-S2 amino acids had no effect on the infectivity of HDV (1), it is also highly unlikely that they play an essential role for HBV entry. Together, this excludes any essential contribution of the pre-S2 domain as a whole or even only part of it, e.g., a putative TLM within, for virus entry or membrane penetration. Moreover, all assembly competent pre-S2 mutants were highly susceptible against inhibition of the infections in HepaRG cells or PHH with the previously described myristoylated pre-S1-derived lipopeptide HBVpre-S/2-48myr. This indicates that none of these mutants can bypass the authentic pre-S1 receptor-mediated entry pathway.

Since introduction of deletions exceeding 20 amino acids within the pre-S2 region resulted in a progressive impairment of virion assembly, the role of the pre-S2 domain in the L protein is probably related to a spacer function which might assure an appropriate distance between the nucleocapsid binding site and the TM-1/sig-1 of the S domain. This issue has been addressed by Kluge et al., who reported that a deletion in the L protein comprising most of the pre-S2 domain and in addition 60 amino acids of the S domain (containing TM-1/sig-1) was compatible with virus assembly (19). This apparent contrast to our results may be explained by the assumption that the first hydrophobic region in the S domain of the L-protein (TM-1/sig-1) is present in our constructs but absent in the constructs of Kluge et al. Thus, the TM-1/sig-1-mediated attachment of the L protein to the membrane may restrict the length of the putative pre-S2 spacer.

The spacer function of pre-S2 in the presence of TM-1/sig-1 does apparently not rely on the formation of distinct structural elements because a randomized sequence is presumably intrinsically unfolded. In contrast, the effects on infectivity that we observed upon replacement of the 30 amino acids with the corresponding polymerase sequence after frameshift in the mutant S114-143 (Fig. 3B) might be explained by a reduced structural flexibility that disables the ability of the pre-S domains of L protein to posttranslationally trespass the virus membrane.

As shown in Fig. 2, pre-S2 deletions in the L protein larger than 20 amino acids resulted in a drastically diminished or even abrogated virion assembly competence. However, since the shortened L proteins are expressed in the transfected HuH-7 cells, we hypothesize that they are retained in the endoplasmic reticulum of the cell, where they possibly accumulate as has been previously described for similar mutants. On the other hand, the L M+ genomes demonstrated a sustained replication in PHH after infection with virions that were transcomplemented with the pre-S2 scrambled L protein (Fig. 4E). Thus, to establish a persistent infection, an intact pre-S2 region is not necessary, either as a constituent of the entering virus or as a part of newly expressed envelope proteins in the early phase after entry, e.g., by activation of signal transduction pathways.

This leads to some important clinical implications. In patients suffering from chronic HBV infection, mutants with even large deletions of pre-S2 frequently rise and may become the dominating virus subpopulation, especially after lamivudine therapy (29, 32). We conclude from our results that these mutants must parasitize other HBV subpopulations. The pre-S2 deletion mutants might have a selective advantage, e.g., due to a higher replication rate (26) or an immune escape mechanism (37), but for particle secretion they clearly depend on helper genomes, which provide functional L proteins competent for mediating nucleocapsid envelopment. Conversely, after entering an HBV-naive hepatocyte these genomes would
be trapped, leading to the intracellular accumulation of the altered envelope proteins. Their inability to promote virus secretion could contribute to the development of ground-glass hepatocytes (11). At least one defined subtype of this histopathological change is tightly associated with pre-S2 deletion mutants and was described as a lesion preceding hepatocellular carcinoma (36).

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REFERENCES