Reduced Secretion of Virions and Hepatitis B Virus (HBV) Surface Antigen of a Naturally Occurring HBV Variant Correlates with the Accumulation of the Small S Envelope Protein in the Endoplasmic Reticulum and Golgi Apparatus

Pong Kian Chua,¹ Robert Yung-Liang Wang,¹ Min-Hui Lin,¹ Tetsuya Masuda,¹
Fat-Moon Suk,^{1,2} and Chiaho Shih^{1*}

Institute for Human Infections and Immunology, Department of Pathology, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555-0609,¹ and Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan²

Received 17 May 2005/Accepted 4 August 2005

We identified two novel naturally occurring mutations (W74L and L77R) in the small S envelope protein of hepatitis B virus (HBV). Mutation L77R alone resulted in >10-fold-reduced secretion of virions. In addition, the 2.8-fold reduction of the extracellular HBV surface antigen (HBsAg) of mutant L77R from transfected Huh7 cells appeared to be correlated with a 1.7-fold reduction of intracellular HBsAg, as measured by enzyme-linked immunosorbent assay (ELISA). Surprisingly, opposite to the ELISA results, Western blot analysis revealed a near-10-fold-increased level of the intracellular mutant small S envelope protein. The discrepancy between ELISA and Western blot data was due to significant accumulation of the mutant L77R HBsAg in the intracellular pellet fraction. In contrast to HBsAg, the secretion of HBeAg was normal in L77R-transfected cells. The wild-type HBsAg was usually more diffuse and evenly distributed in the cytoplasm, often outside the perinuclear endoplasmic reticulum (ER) and Golgi apparatus, as observed by immunofluorescence assay. In contrast, the L77R mutant HBsAg tends to be highly restricted within the ER and Golgi, often accumulated in the Golgi compartments distal from the nucleus. The almost exclusive retention in the ER-Golgi of L77R HBsAg was similar to what was observed when the large envelope protein was overexpressed. These multiple aberrant phenotypes of mutant L77R can be corrected by a second naturally occurring S envelope mutation, W74L. Despite the accumulation of L77R HBsAg in ER-Golgi of transfected Huh7 cells, we detected no increase in Grp78 mRNA and proteins, which are common markers for ER stress response.

Hepatitis B virus (HBV) is a major human pathogen. Chronic infection with HBV leads to the development of cirrhosis and hepatocellular carcinoma (2, 16, 36). HBV variants are often found in chronically infected patients (19, 37). The most common naturally occurring mutation in human HBV core protein is at amino acid (aa) 97, changing a highly conserved isoleucine (HBsAg subtype *adr*) or phenylalanine (HBsAg subtype *ayw*) to a leucine (L) (3, 12–15, 20). In contrast to the established dogma of preferential virion secretion of mature genome for wild-type (WT) hepadnaviruses (17, 33, 40, 44, 47, 48), the 97L mutation results in secretion of virions containing an immature genome into the medium and is characterized by excessive amounts of minus-strand DNA (47, 48). Even though the immature secretion phenotype has been observed with woodchuck and snowgoose hepadnaviruses (7, 42), it has not been reported with human patients. This may be due to the presence of naturally occurring compensatory mutations for 97L in the core protein at positions 5 (11) or 130 (49), both changing a highly conserved proline to threonine.

HBV surface antigens (HBsAg) consist of three structurally related large (L), middle (M), and small (S) envelope proteins. These proteins share a common carboxyl terminus, with the L protein containing pre-S1, pre-S2, and small S domains, and the M envelope protein containing pre-S2 and small S domains. The small S protein is expressed at high levels and can be secreted independently of L and M envelope proteins. Both L and S envelope proteins are needed for virion secretion, while M protein is dispensable (4, 43). Furthermore, overexpression of pre-S1 containing L protein blocks HBsAg secretion (8, 31, 32, 34). It is generally believed that proper stoichiometry between L and S envelope proteins is important for secretion of HBsAg and virions. However, no similar phenotype is observed when the small S protein is overexpressed, probably because the small S envelope protein is already in large excess to the L envelope protein in the normal setting of wild-type HBV replication.

The blockage of secretion of HBsAg by the overexpressed L envelope protein results in the accumulation of HBsAg in the endoplasmic reticulum (ER) lumen, which in turn can induce ER stress (46). In transgenic mice, the intracellular retention of HBsAg in hepatocytes can cause pleiotropic physiological changes, ground glass morphology (8, 9), and hypersensitivity to inflammatory cytokines (18). To date, it remains unclear if the retention of HBsAg in ER in experimental

* Corresponding author. Mailing address: Department of Pathology and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-0609. Phone: (409) 772-2563. Fax: (409) 747-2429. E-mail: cshih@utmb.edu.

TABLE 1. Clinical data and natural occurrence of HBcAg codon 97 mutation in 20 patients with chronic hepatitis B^a

Patient no.	Age (yr)	Sex	HBsAg	HBeAg	Anti-HBe	ALT (IU/liter) ^b	Amino acid at HBcAg codon 97 ^c
1	42	M	+	+	—	228	L
2	34	M	+	+	—	28	I>L
3	27	F	+	+	—	56	I
4	28	M	+	+	—	366	L>I
5	37	M	+	+	—	135	L
6	44	F	+	—	+	17	L>I, F ^d
7	29	M	+	—	+	51	I
8	37	M	+	—	+	45	L>I
9	16	M	+	+	—	69	L>I
10	57	M	+	—	+	48	I
11	47	M	+	—	+	43	I>L, F ^d
12	29	M	+	—	+	96	I>L
13	59	M	+	—	+	175	L>I
14	20	M	+	—	+	33	I>L
15	53	F	+	—	+	40	I>L
16	43	M	+	—	+	71	L>I
17	56	M	+	+	—	75	L>I, F ^e
18	23	F	+	—	+	30	L
19 ^f	20	M	+	+	+	54	I
20	29	M	+	+	—	81	I

^a +, above the cutoff; —, below the cutoff. Samples were assayed for HBsAg, HBeAg, and anti-HBe antibody as described in Materials and Methods. M, male; F, female.

^b Serum samples were assayed for alanine aminotransferase (ALT) (in international units per liter). The normal upper limit is approximately 40 IU/liter.

^c See Materials and Methods for DNA extraction, cloning, and sequencing details. At least five independent clones were sequenced. In the case of mixtures of WT and mutant 97, I>L indicates more clones having I97I than I97L. Similarly, L>I indicates that the mutant was predominant over WT.

^d Both Leu and Phe were identified at codon 97.

^e One clone contained Val at codon 97.

^f Patient 19 was both HBeAg and anti-HBe positive and was probably screened at the stage of seroconversion.

models can actually occur in natural infection with or without overexpression of the L envelope protein.

The small S envelope protein contains two domains with highly frequent, naturally occurring mutations (37, 41). One domain (amino acids 28 to 51) coincides with a T-cell epitope (10), while the other domain (amino acids 124 to 148) coincides with a B-cell epitope. The latter, known as the group *a* determinant, confers the protective virus-neutralizing epitopes (16). In addition to these two domains mapped by genetic or immunological approaches, there is another putative core-envelope interaction domain, which spans amino acids 56 to 80. This domain is important for the secretion of virions (29). Naturally occurring mutations in the group *a* determinant, which is located in the lumen of the endoplasmic reticulum, have been found to exhibit reduced secretions of virions or HBsAg (21, 22, 23). However, naturally occurring mutations in the core-envelope interaction domain, which is on a cytosolic loop of the small S envelope protein, have not been functionally characterized.

Previously, an artificial mutation (A119F) in the core-envelope interaction region of the pre-S1 domain was shown to suppress the immature secretion phenotype caused by the core 97L mutation (27). In our search for other naturally occurring envelope mutations that can suppress the immature secretion phenotype, we discovered a low-level secretion small S envelope mutant, L77R, and its compensatory mutation, W74L. The L77R mutant exhibited multiple phenotypes: (i) significantly reduced levels of secreted virions, (ii) reduced levels of intracellular and extracellular HBsAg by enzyme-linked immunosorbent assay (ELISA), (iii) increased accumulation of intracellular small S envelope protein, (iv) enhanced intracellu-

lar accumulation of full-length relaxed circular (RC) form DNA, and (v) retention of HBsAg in the endoplasmic reticulum and Golgi. All of these aberrant phenotypes can be rescued simultaneously by a naturally occurring mutation W74L, which is only 3 aa away from the mutation L77R.

MATERIALS AND METHODS

Serum samples from chronic hepatitis B patients. Twenty serum samples were obtained from Taipei Medical University Hospital, Taipei, Taiwan. The patients' clinical data are summarized in Table 1. The conventional serology data include HBsAg, HBeAg, anti-HBe, and alanine aminotransferase. Amino acid sequences at position 97 of HBV core antigen are summarized in Table 1.

DNA extraction from serum samples. Approximately 200 to 300 μ l of serum samples from HBV patients was subjected to sucrose cushion and cesium chloride gradient ultracentrifugation. Collected fractions corresponding to Dane particles (around 1.24 g/cm³) were pooled and dialyzed against 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Core-associated HBV DNA was then extracted by proteinase K digestion, followed by phenol-chloroform extraction and isopropanol precipitation.

PCR cloning and sequencing. For PCR amplification of core and envelope genes, HBV DNA was extracted from 100 μ l of serum by proteinase K digestion overnight in lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 1.2- μ g/ μ l proteinase K) at 50°C, followed by phenol-chloroform extractions and ethanol precipitation. The DNA pellets were resuspended in 20 μ l of 10 mM Tris-HCl (pH 8.0) buffer.

The entire core gene (*ayw* numbering system; nucleotide position 1861 to 2478) was amplified in a 50- μ l reaction mixture consisting of the following: 10 μ l of extracted DNA, 1.6 μ l of a 1.25 mM deoxynucleoside triphosphate (dNTP) mixture, 5 μ l of 10 $\times$ PCR buffer, 1 μ l each of 250-ng/ μ l forward (1861S, 5'-ACTGTTCAAGCCTCCAAGCT-3') and reverse (2478AS, 5'-TCCCACCTTATGAGTCCAAG-3') primers and 1.25 U of TaqI polymerase. The reaction was performed for 30 cycles, each consisting of 94°C for 10 s, 55°C for 10 s, and 72°C for 1 min. The entire envelope gene was similarly amplified using forward (2459S, 5'-CCTTGGAAGTCAAGGT-3') and reverse (988AS, 5'-ACTTTC CAATCAATAG-3') primers and 1.25 U of TaqI polymerase. The reaction was

TABLE 2. Oligonucleotides used for PCR mutagenesis

Oligonucleotide ^a	Sequence ^b
W74L (S).....	5'-TGTCTGTTATCGCT T GATGTGTCTGCGG-3'
W74L (AS).....	5'-CCGCAGACACATC A AGCGATAACCAGGACA-3'
L77R (S).....	5'-TATCGTGGATGTGT C GCGCGCGTTTATC-3'
L77R (AS).....	5'-GATAAACGCCG C CGACACATCCAGCGATA-3'
W74L/L77R (S).....	5'-GGTTATCGCT T GATGTGT C GCGCGCGTTT-3'
W74L/L77R (AS).....	5'-AAAACGCCG C CGACACATC A AGCGATAACC-3'

^a S, sense polarity; AS, antisense polarity.

^b Mutated codons are underlined; mutation sites are in boldface type.

performed for 30 cycles, each consisting of 95°C for 10 s, 45°C for 10 s, and 72°C for 50 s. A seminested PCR was performed with a second forward primer (2719S, 5'-AGTTAATCATTACTTCCAAAC-3') and the same reverse primer (988AS, 5'-ACTTTCCAATCAATAG-3') using 10 µl of the product from the first round of amplification. PCR products were then gel purified and cloned into pGEMT-EZ vectors (Promega, Wisconsin). At least five independent clones from each serum sample were selected and sequenced through the positions corresponding to core amino acids 5, 97, and 130. At least four clones for pre-S1 and small S envelope genes of sample 17 were sequenced with the Sequenase kit (United States Biochemical Corp., Cleveland, OH). While amino acids 50 to 90 and 110 to 160 of the small S envelope gene from sample 17 were sequenced, only the putative core-envelope interaction region (aa 98 to 119) of the pre-S1 domain was sequenced.

Preparation of the glucose-regulated protein (Grp78) probe. To prepare the Grp78 probe, total RNA was isolated from Huh7 cells treated with tunicamycin (50 µg/ml) for 16 h. One microgram of total RNA was used for cDNA synthesis in a 20-µl reaction mixture at 37°C for 1 h, containing 0.5 µg of oligo(dT), 10 mM dithiothreitol, 0.5 mM each dNTP, 5× First Strand buffer, and 100 U Moloney murine leukemia virus reverse transcriptase (First Strand cDNA Synthesis kit; Novagen, Wisconsin). A 5-µl aliquot of the cDNA reaction product was amplified in a 50-µl PCR mixture consisting of 1.25 mM dNTP, 5 µl 10× PCR buffer, 1 µl (each) of 100-ng/µl forward (PubMed accession number, NM 005347; S416, 5'-GAAGGGGAACGCTCTGATTGGCGAT-3') and reverse (AS1460, 5'-ACA TCAAGCAGTACCAAGTCACT-3') primers, and 1.25 U of TaqI polymerase. The reaction was performed for 30 cycles, each consisting of 94°C for 10 s, 50°C for 10 s, and 72°C for 1 min. The 1.1-kb PCR amplicon was cloned into pGEMT-EZ (Promega, Wisconsin) and sequenced to confirm the identity of Grp78 DNA. The vector-free Grp78 DNA fragment was radiolabeled and used as a probe in Northern blot analysis.

Plasmids. Plasmid pECE24 encodes the wild-type small S envelope protein (HBsAg subtype *adw*) under the transcriptional control of the simian virus 40 (SV40) early promoter (32). This plasmid pECE24 was used as the template for mutagenesis to create mutations W74L and L77R and the double mutant W74L/L77R, with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used for mutagenesis are shown in Table 2. Plasmid WT SK/O (HBsAg subtype *ayw*) contains a WT HBV genomic dimer with an ablated AUG of the small S gene, while plasmid F97L S K/O (HBsAg subtype *ayw*) contains an HBV genomic dimer with an F97L mutation in the core gene and an ablated AUG of the small S gene (47). All mutants were confirmed by DNA sequencing.

Transfection, core particle isolation, and core particle-associated HBV DNA extraction. Wild-type or mutant envelope expression vectors (W74L, L77R, or W74L/L77R) were cotransfected with F97L S K/O into Huh7 cells by the calcium phosphate method. Cell culture media were collected on days 5 and 7 and subjected to sucrose cushion and cesium chloride gradient ultracentrifugation to separate virions from naked core particles as previously described (47). Intracellular core particles were isolated as previously described (47). Core-associated HBV DNA was extracted by phenol-chloroform:isoamyl alcohol and precipitated with ethanol as previously described (47).

Southern and Northern blot analyses. Southern blotting was performed on core particle-associated DNA using a 3.1-kb HBV-specific probe as previously described (47). RNA was extracted from Huh7 cells 3 days posttransfection or 16 h after treatment with 50-µg/ml tunicamycin (stock of 5 mg/ml; Sigma Chemical Co., St. Louis, MO) using TRI Reagent (39) (Sigma-Aldrich, St. Louis, MO). Thirty micrograms of total RNA was used for Northern blot analysis with a 3.1-kb HBV-specific probe or a 1.1-kb Grp78-specific probe.

HBsAg and HBeAg ELISA. The ELISA kits for HBeAg, anti-HBe, and HBsAg in the patients' sera were from the AXSYM system (Abbott Diagnostic Division, Germany). The ELISA kits for HBsAg and HBeAg in the

conditioned media and intracellular lysates were from International Immunodiagnostics Co., Foster City, CA. Cell culture media were collected and analyzed for secreted HBsAg and virions. To isolate intracellular HBsAg and HBeAg from transfected Huh7 cells, approximately 6×10^6 cells were scraped off one 10-cm dish in 1 ml of phosphate-buffered saline (PBS) and spun down by microfuge for 5 min at 5,000 rpm. The pellet was resuspended in 200 µl of PBS and subjected to five cycles of freeze-thawing. The freeze-thawed cell lysates were then centrifuged at 13,000 rpm for 5 min. The resulting supernatant was collected and analyzed for HBsAg or HBeAg by ELISA or stored at -20°C for Western blot analysis of small S envelope proteins (see below). The freeze-thawed pellet fraction was stored at -20°C and used for Western blot analysis of small S envelope proteins (see below).

Western blotting. Protein samples were prepared from (i) total cell lysates, (ii) the supernatant, and (iii) the pellet from the cell lysates after multiple freeze-thaws. For the total cell lysate, 200 µl of 2× protein lysis buffer (4% SDS, 100 mM Tris-HCl [pH 6.8], 0.2% bromophenol blue, 5% 2-mercaptoethanol, 20% glycerol) was used for 6×10^6 Huh7 cells in one 10-cm dish. The freeze-thaw supernatant and pellet were prepared as described above. Ten-microliter aliquots of the supernatant fraction were mixed with 2× protein lysis buffer before being loaded on each lane of an SDS-polyacrylamide gel electrophoresis (PAGE) gel. The freeze-thawed pellet fraction was resuspended in 100 µl of 2× protein lysis buffer and resulted in a final volume of approximately 200 µl. A 20-µl aliquot of the resuspended pellet fraction was loaded on each lane of the SDS-PAGE gel.

Protein samples were boiled for 5 min, chilled on ice, and subjected to SDS-PAGE and Western blotting with a variety of antibodies (Abs), including a mouse monoclonal antibody (MAb) specific for small S group α determinant (Hyb-5124A; Institute of Immunology, Tokyo, Japan), a rabbit anti-core polyclonal antibody (1), a rabbit anti-Grp78 antibody (Santa Cruz Biotech., Santa Cruz, CA), and a mouse anti-tubulin antibody (Sigma, St. Louis, MO). Visualization of proteins was by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence analysis and antisera. For immunofluorescent staining, cells were cultured on noncoated glass coverslips. Three days posttransfection, cells were rinsed with PBS twice, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 30 min, and incubated in 2% blocking buffer (Roche, Indianapolis, IN) for 1 h. The cells were then incubated sequentially with primary and secondary antibodies, as follows. HBsAg was stained with diluted (1:500) goat anti-HBsAg antibody (Dako Cytomation Co., Carpinteria, CA). A cell surface marker integrin (CD29) was stained with diluted (1:200) mouse anti-CD29 polyclonal antibody (Beckman Coulter, Fullerton, CA). An endoplasmic reticulum marker, calnexin, was stained with diluted (1:200) rabbit anti-calnexin polyclonal antibody (StressGen Biotechnologies Co, Victoria, Canada). A Golgi marker, G58 K protein, was stained with diluted (1:200) mouse anti-G58 K MAb (Sigma, St. Louis, MO). The ER stress-specific marker, Grp78, was stained with diluted (1:25) rabbit anti-Grp78 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA). Donkey anti-goat tetramethylrhodamine isocyanate, donkey anti-rabbit fluorescein isothiocyanate, and rabbit anti-mouse fluorescein isothiocyanate (all from Santa Cruz Biotech, Santa Cruz, CA) were used as the secondary antibodies for some experiments (see the results shown in Fig. 7 to 9). The nuclei of the cells were counterstained with 10 µg/ml of 4',6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). After being immunostained, the coverslips were mounted on slides in Gelvatol medium (20% polyvinyl alcohol in 10 mM Tris-HCl [pH 8.6]). Images were collected with a Zeiss confocal microscope (LSM 510) and processed with Photoshop.

Statistical analysis. Student's *t* test was employed to calculate the *P* values of HBsAg between the wild-type pECE24 and mutant L77R.

RESULTS

Frequent core 197L mutation in vivo. It is dogma that the mature hepadnaviral genome is preferentially secreted over the immature genome in vivo and in vitro (17, 33, 40, 44, 47, 48). An exception to this is a highly frequent, naturally occurring HBV core mutant 97L with an immature secretion phenotype in vitro (47, 48). To determine if immature secretion could also be found in chronically infected patients, we screened 20 serum samples by PCR cloning and DNA sequencing analysis (Table 1). Among these 20 samples, 5 samples contained only isoleucine-97, and 3

FIG. 1. No immature secretion phenotype was identified in eight different HBV-positive serum samples by gradient centrifugation and Southern blot analysis. HBV particles present in 200- to 300- μ l aliquots of serum samples were pelleted through a sucrose cushion before further purification through a 20 to 50% (wt/vol) cesium chloride gradient centrifugation. For each serum sample, collected fractions corresponding to different buoyant densities were dialyzed to remove CsCl before DNA extraction. Fractions corresponding to the density of virion particles (around 1.24 g/cm³) were pooled together before dialysis. The isolated HBV DNAs from each fraction were subjected to Southern blot analysis. Density fractions containing no detectable HBV DNA by Southern blot were deleted and not shown in this composite figure. The HBV core gene-specific DNAs from all samples were sequenced, and only samples 9, 17, and 18 (asterisks) were found to predominantly contain the I97L mutation (Table 1). This I97L mutation, in the absence of compensatory mutations, is known to be necessary and sufficient for the virion secretion of immature genome (47). Typical immature and mature secretion phenotypes observed in cell culture are shown (left). RC, relaxed circular; SS, single-stranded HBV DNA replicative intermediates.

contained only leucine-97. The remaining 12 samples contained mixtures of both. Seven samples (7/12) contained more leucine-97 than isoleucine-97, and 5 samples (5/12) contained more isoleucine-97 than leucine-97. Taken together, 15/20 samples (75%) contained a detectable leucine-97 mutation, a result consistent with previous reports that 97L is a highly frequent mutation (3, 12–15, 20).

No immature secretion phenotype was found in patients' sera. Among a total of 20 HBsAg-positive serum samples (Table 1), 12 samples contained either insufficient serum or low levels of viral DNA and could not be used for gradient centrifugation and Southern blot analysis (data not shown). HBV DNA was extracted from gradient-purified virion particles isolated from the remaining eight samples (Materials and Methods). As shown in Fig. 1, these viral DNAs were subjected to Southern blot analysis. All of the samples contained RC DNA of a mature genome, while samples 17, 18, and 20 exhibited additional trace amounts of immature genome of single-strand (SS) DNA. None of the samples displayed excessive amounts of SS DNA in the secreted virions characteristic of immature secretion (47, 48).

Presence of a core I97L mutation is not sufficient for immature secretion in vivo. As shown in Fig. 1, sequence analysis of the core gene from serum samples 9, 17, and 18 revealed the presence of a 97L mutation, but not compensatory P5T or P130T mutations (references 11 and 49 and data not shown). Based on all the published HBV core 97L mutant sequences, compensatory core mutations P5T or P130T are associated with approximately 76% of the 97L mutants (11). Previously, we demonstrated by a genetic approach that the core-envelope interaction is important for virion secretion (27). Taken together, this suggested that there may be some as-yet-unknown compensatory mutations in HBV envelope proteins of samples 9, 17, and 18, particularly within the putative core-envelope interaction domains of the envelope proteins (5, 6, 28, 29).

Because serum samples 9 and 18 were exhausted for other experiments, we turned our focus to sample 17.

Identification of small S envelope mutations W74L and L77R. We examined the putative pre-S1 and small S core-envelope interaction domains from sample 17 by PCR cloning and sequence analysis. We identified two small S gene mutations at aa 74 (tryptophan to leucine; W74L), and 77 (leucine to arginine; L77R) in four of four bacterial clones. As depicted in the topological model of Fig. 2, both mutations are located in a cytosolic loop previously proposed to interact with capsids (29). Both mutations are silent in the overlapping polymerase gene.

A low-level virion secretion phenotype of the small S envelope mutant L77R can be rescued by the mutation W74L. To functionally test if these small S mutations (W74L and L77R) were indeed compensatory for the immature secretion of mutant 97L, we introduced these mutations into a small S enve-

FIG. 2. A topological model of HBV small S envelope protein (38) and naturally occurring mutations W74L and L77R are shown. The core-envelope interaction domain and group *a* determinant are highlighted.

FIG. 3. A naturally occurring small S envelope mutation L77R was identified from sample 17. This mutation, by itself, results in low-level virion secretion, which can be rescued by another envelope mutation, W74L. A pECE24 expression vector of the small S envelope protein is under the transcriptional control of an SV40 early promoter (32). Plasmid pECE24 was engineered to contain single mutations W74L and L77R or double mutation W74L/L77R, respectively (sequencing data not shown). These mutant pECE24 plasmids were cotransfected into Huh7 cells with the HBV tandem dimer containing core 97L mutation and S gene AUG K/O (F97L SK/O) (47). (a) Southern blot analysis of virion DNA present in cell culture media from transfected Huh7 cells. Virions were purified via sucrose cushion and cesium chloride gradient ultracentrifugation. (b) HBV intracellular core-associated DNA from transfected cell lysates was subjected to Southern blot analysis with a 3.1-kb HBV-specific probe. The arrowhead highlights the enhanced accumulation of the full-length RC form in mutant L77R, which was observed in three independent experiments.

lope protein expression vector, pECE24 (32), either individually or in combination. The mutant plasmid pECE24 carrying mutations W74L or L77R was cotransfected into Huh 7 cells with the plasmid F97L S K/O, which is a genomic dimer deficient in the synthesis of small S protein (47). Media from these cotransfected cultures were processed by sucrose cushion and cesium chloride gradient ultracentrifugation to separate virions from naked core particles. The naked core particles were discarded. Virion-associated HBV DNA was then extracted and analyzed by Southern blotting.

As shown in Fig. 3a, lane 1, plasmid WT SK/O exhibited an approximately fourfold-stronger signal intensity of the nascent RC form than that of SS DNA replicative intermediates when wild-type envelope protein was provided *in trans* by cotransfection with plasmid pECE24. In contrast, Fig. 3a, lane 2, shows the genomic dimer of mutant F97L SK/O secreted virions containing almost equal intensities between RC and SS forms, a feature characteristic of immature secretion (47). When plasmid pECE24 was replaced by plasmid W74L or W74L/L77R, we observed no rescue of the immature secretion phenotype (i.e., the ratio between the RC and SS DNAs shown in Fig. 3a, lane 3 or 4, remained similar to that shown in lane 2).

While we found no compensatory envelope mutants for the immature secretion of core mutant F97L, we noted that mutant L77R resulted in a low-level virion secretion phenotype (Fig. 3a, lane 5), despite its efficient intracellular viral DNA replication (Fig. 3b, lane 10). A similar phenotype was previously observed with core mutants (24, 26, 35), large L envelope mutants (27, 30), and small S envelope mutants (22, 23). This low-level virion secretion phenotype of mutant L77R (Fig. 3a,

lane 5) was not observed in mutant W74L/L77R (Fig. 3a, lane 3), indicating a compensatory nature of mutation W74L. Consistent with our previous studies, plasmid F97L SK/O exhibited a reduced level of intracellular RC DNA replicative intermediates (47).

Accumulation of full-length RC form in mutant L77R can be rescued by the mutation W74L. We also noted the preferential accumulation of full-length RC-form DNA (Fig. 3b, lane 10) in cells cotransfected with plasmids L77R and F97L S K/O. Accumulation of full-length RC DNA in an SK/O mutant deficient in small S protein synthesis has been observed previously (47), as well as in a pre-S1 deletion mutant truncated with the pre-S2/S promoter (30). This is likely due to a compromised ability of host cells to secrete mutant virions. As shown in Fig. 3b, lane 8, the accumulation of full-length RC form in mutant L77R was not observed in double mutant W74L/L77R, indicating that the mutation W74L can suppress the phenotype of mutant L77R.

A low-level HBsAg secretion mutant, L77R, can be suppressed by another envelope mutant, W74L. Low level-secretion of mutant L77R is not limited to HBV virions released into the medium. As shown in Fig. 4a, culture media from transfected cells were tested for secreted HBsAg by ELISA. Again, the level of HBsAg in medium collected from Huh7 cells transfected with the small S envelope mutant L77R expression vector was significantly reduced compared to that of wild-type pECE24, double mutant W74L/L77R, and single mutant W74L (Fig. 4a). These results demonstrated that mutant W74L is dominant over mutant L77R, not only in the secretion of virions, but also with HBsAg.

FIG. 4. The small S envelope mutation L77R by itself results in lower levels of both extracellular and intracellular HBsAg but not HBeAg. This phenotypic effect of mutation L77R can be rescued by another envelope mutant, W74L. Cell culture medium (a) or total cytosolic proteins (b) were harvested from Huh7 cells cotransfected with either wild-type or mutant pECE24 plasmids and F97L SK/O. HBsAg was analyzed by the commercial ELISA kit (see Materials and Methods). *, $P < 0.05$ between wild-type pECE24 and mutant L77R from five independent experiments. (c) HBeAg from cell culture medium (left) or total cytosolic proteins (right) was harvested from four independent transfections and analyzed by a commercial ELISA kit. The y axis scale is a ratio of sample optical density at 450 nm and the cutoff value, based on the negative controls provided by the commercial ELISA kit.

Intracellular HBsAg of L77R-transfected cells was also reduced by ELISA. To elucidate the mechanism of reduced levels of secreted virions and HBsAg associated with mutant L77R, we examined the intracellular steady-state level of HBsAg. We lysed the cells by repetitive freeze-thaws and harvested the supernatant by centrifugation. We then compared the amounts of HBsAg in the supernatants between transfections with single mutant L77R or wild-type pECE24 plasmids. As shown in Fig. 4b, mutant L77R appeared to contain a 1.7-fold-reduced level of intracellular HBsAg by ELISA, relative to that of the wild-type pECE24 plasmid.

Secretion of HBeAg was not reduced in cells transfected with mutant L77R. As shown in Fig. 4c, the readings of HBeAg in the media (left) or intracellular lysates (right) harvested from cotransfections with either the genomic dimer plasmid F97L SK/O and WT pECE24 versus mutant L77R were very similar in four independent experiments. This results suggest that the functions of ER and Golgi in host cells cotransfected with plasmid F97L SK/O and mutant L77R remained normal, despite the fact that the HBsAg secretion of mutant L77R was reduced (Fig. 4a). Consistent with this result was the fact that we observed no intracellular accumulation of secretory hepatic markers, such as α -1-antitrypsin and transferrin (data not shown). Our results shown in Fig. 4c also confirmed that the decreased levels of intracellular and extracellular HBsAg

FIG. 5. The reduced level of HBsAg protein produced from small S envelope mutant L77R is not caused by decreased expression of HBsAg-specific mRNA. Total RNA was extracted from Huh7 cells 3 days posttransfection with SV40 expression vector plasmids containing either a wild-type S envelope protein from pECE24 (32) or its derived mutant, L77R. Thirty micrograms of total RNA was analyzed by Northern blotting with a 3.1-kb HBV-specific probe (39). The 18S and 28S ribosomal RNAs from the cell lysates of the transfected culture were visualized by ethidium bromide staining on the agarose gel and served as an internal control for the RNA sample loadings (bottom). An RNA ladder was included as a size marker (data not shown).

(Fig. 4a and b) were not due to fluctuations in transfection efficiency.

The lower level of intracellular HBsAg protein from mutant L77R is not due to a reduced level of HbsAg-specific RNA. The possibility that the decreased level of the HBsAg protein from mutant L77R could be due to reduced levels of the small S envelope-specific mRNA was examined by Northern blot analysis. However, as shown in Fig. 5, similar levels of the small S-specific mRNA were detected between wild-type pECE24 and mutant L77R.

A lower level of intracellular HBsAg from mutant L77R by ELISA is correlated with a higher level of HBsAg in the pellet fraction by Western blot analysis. To confirm the ELISA data of the intracellular HBsAg in mutant L77R-transfected Huh7 cells (Fig. 4b), we tested several commercially available Abs for HBsAg by Western blot analysis. We found an anti-S MAb (Hyb-5124A; Institute of Immunology, Tokyo, Japan) that was adequate for Western blot analysis of HBsAg. However, using this MAb, we obtained unexpected Western blot results that were exactly opposite to our previous ELISA data (Fig. 4b). As shown in Fig. 6a, the intracellular amount of the small envelope protein of mutant L77R was at least 10-fold higher than that of the wild-type pECE24 expression vector. A similar result was obtained when the S envelope deficient genomic

FIG. 6. Intracellular accumulation of mutant L77R small S envelope protein in the pellet fraction was observed in transfected Huh7 cells by Western blot analysis using a MAb against the group *a* determinant. The results shown in this figure are from two independent transfection experiments. (a) Total cell lysates from Huh7 cells transfected with mutant L77R small S expression vector exhibited increased levels of both unglycosylated (24-kDa) and glycosylated (27-kDa) small S envelope proteins. Plasmid pECE24 is the parental wild-type counterpart of mutant L77R. (b) An increased level of the small S envelope protein was detected in the total cell lysate of cotransfected Huh7 cells by Western blot analysis. Cotransfection was conducted using the small S envelope-deficient genomic dimer (plasmid F97L SK/O) and the

dimer (plasmid F97L SK/O) was provided in *trans* with either the wild-type (pECE24) or mutant L77R small S envelope expression vectors (Fig. 6b). The ELISA procedure involved repetitive freeze-thaws of transfected Huh7 cells, followed by centrifugation and the use of the supernatant fraction for assay. This unexpected discrepancy between the results from ELISA and Western blotting led us to hypothesize that the majority of the intracellular L77R small S envelope protein was not in the supernatant after centrifugation.

To test this hypothesis, we repeated the same cotransfection experiment as that shown in Fig. 6b and performed Western blot analysis using the redissolved pellets of freeze-thawed cell lysates (which were normally discarded during our routine ELISA procedure). Indeed, consistent with the results using total cell lysates shown in Fig. 6a and b, we found significantly increased level of the small S envelope protein in the pellet fraction of mutant L77R lysates (Fig. 6c). In contrast, the supernatant fraction from mutant L77R contained a lower level of the small S envelope protein (Fig. 6d), which was entirely consistent with our previous ELISA results (Fig. 4b). This result (Fig. 6d) also suggested the possibility that the reduced intracellular level of the L77R HBsAg by ELISA was not due to an altered antigenic conformation which could no longer be recognized by the antibodies used in the commercial ELISA kit. In Fig. 6a to d, asterisks indicate nonspecific cross-reactive cellular proteins, which served as convenient internal controls for equal amounts of sample loading. When the same filters used in the experiments shown in Fig. 6c and 6d were stripped of the anti-S MAb and reprobed with rabbit anti-core polyclonal antibody (1), similar levels of HBV core proteins were found between cotransfections with wild-type pECE24 versus mutant L77R. Again, these results confirmed equal loading of samples during SDS-PAGE.

As shown in Fig. 6d in the case of mutant L77R, we could see a faint band comigrating with the WT gp27, in addition to a faint band migrating in between the WT gp27 and p24. This was not due to gel artifact, since it was highly reproducible in

wild-type (pECE24) or mutant L77R small S expression vector. (c) An increased level of the small S envelope protein was detected in the freeze-thaw pellet fraction of cotransfected Huh7 cells by Western blot analysis. The pellet fraction of cotransfected Huh7 cells was resuspended after centrifugation of the cell lysate prepared by five repetitive freeze-thaws. The same filter was stripped of the anti-S MAb and reprobed with a rabbit anti-core polyclonal antibody. The similar intensity of core protein in different samples indicates that equal amounts of samples were loaded in a SDS-PAGE gel. (d) A reduced level of the small S envelope protein was detected in the supernatant fraction of mutant L77R-cotransfected Huh7 cells by Western blot analysis. An aberrant faint band migrating between the WT gp27 and p24 was noted. While the exact cause of this aberrant banding remains unclear, it was clearly not due to gel artifact, since it was highly reproducible in three independent experiments (data not shown). The similar intensity of core protein in different samples indicates that equal amount of samples were loaded in SDS-PAGE. Asterisks indicate nonspecific and cross-reactive cellular proteins, which are included here as an internal reference for sample loading. These nonspecific bands are present in nontransfected Huh7 cells (data not shown). In addition, they are present in the results shown in Fig. 6A, which used the small S expression vector without any L and M envelope genes.

FIG. 7. Mutant L77R HBsAg displays a highly restricted perinuclear staining pattern. Huh7 cells were transiently cotransfected with an HBsAg-deficient genomic tandem dimer plasmid F97L SK/O and each of the four different small S envelope protein expression vectors, pECE24, L77R, W74L, and W74L/L77R. Cells were harvested at 3 days posttransfection, fixed with 4% paraformaldehyde, and stained for HBsAg (red) and a cell surface marker integrin CD29 (green). Because integrin acquires glycosylation in the ER-Golgi, green coloring was also present in the putative ER-Golgi areas. Wild-type pECE24 and mutants W74L and W74L/L77R showed diffuse granular staining which covered the entire cytoplasm, as shown in the merged pictures (c, i, and l). In contrast, mutant L77R displayed a highly restricted perinuclear staining pattern with a lot of empty cytoplasmic space without HBsAg, as shown in the merged picture (f). The blue color of the nucleus was stained with DAPI. Scale bar, 10 μ m.

three independent experiments. We speculate that the retarded mobility of “p24” of mutant L77R is either due to the charge effect caused by the L-to-R substitution at amino acid 77 or due to some unknown aberrant modification of the mutant p24 protein.

Immunofluorescence microscopic examination of the subcellular localization of the small S envelope protein. Although

the results shown in Fig. 6d revealed that the amount of the intracellular L77R small S envelope protein was reduced in the supernatant compared to that of the wild type, it was unclear where the mutant L77R protein was accumulated intracellularly. To further investigate this issue, we examined the subcellular distribution of the mutant envelope proteins via immunofluorescence microscopy. As shown in the first column of

FIG. 8. Colocalizations of HBsAg and an ER-marker calnexin by immunofluorescence microscopy. Huh7 cells were transiently cotransfected with plasmids F97L SK/O and pECE24 or L77R as described in the legend to Fig. 7. Cells were fixed with 4% paraformaldehyde and stained for HBsAg (red) and an endoplasmic reticulum-specific marker Calnexin (green). Consistent with the results in Fig. 7, wild-type HBsAg displayed a more diffuse and granular staining pattern within the cytoplasm. There is a strong correlation between the stainings of HBsAg (red) and calnexin (green), as shown in the merged picture. The blue color of the nucleus was stained with DAPI. Scale bar, 10 μ m.

Fig. 7, no striking difference in the staining patterns of the small S envelope protein (staining red) of wild-type pECE24 (Fig. 7a), mutant W74L (Fig. 7g), and double mutant W74L/L77R (Fig. 7j) was apparent. While these three different viral genotypes shared a common pattern with more diffuse and fine granular staining, mutant L77R (Fig. 7d) appeared to be denser and brighter in staining. However, in some rare cases, denser and brighter staining was also observed in cells transfected with the other three genotypes, especially when the cell size was small and when a higher level of HBsAg was expressed (e.g., see the two cells near the top border of Fig. 8a).

To further distinguish the HBsAg staining patterns between the wild-type and mutant L77R, we marked the cell peripheries with an anti-integrin (CD29) antibody (staining green) (Fig. 7b, e, h, and k). As shown in the merged pictures in the right column of Fig. 7, it became evident that wild-type pECE24 (Fig. 7c), mutant W74L (Fig. 7i), and W74L/L77R (Fig. 7l) often distributed throughout the entire cytoplasm of transfected Huh7 cells. In many cases, HBsAg (red) appeared to be preferentially absent or greatly reduced in staining intensity in perinuclear Golgi-like areas (green) (Fig. 7i and 7l). In contrast, most of the HBsAg (red) of mutant L77R (Fig. 7f) was seen to be highly restricted and concentrated as dense clusters in the perinuclear region, presumably endoplasmic reticulum and/or Golgi compartments of the transfected Huh7 cells (Fig. 8). Unlike wild-type pECE24 and the other mutants, there was plenty of empty cytoplasmic space without HBsAg for mutant L77R (Fig. 7f). Consistent with the previous results shown in Fig. 3 and 4, the W74L mutation appeared to be compensatory for the primary mutation L77R (Fig. 7l).

The mutant L77R but not wild-type HBsAg is colocalized with the Golgi 58k protein. To confirm the subcellular localizations in ER-Golgi, we used antibodies specific for the ER-Golgi markers. As shown in Fig. 8, both wild-type pECE24 and

mutant L77R HBsAg (staining red) were very well colocalized with calnexin (green) in ER. This was consistent with the presence of glycosylated gp27 of the small envelope protein of mutant L77R by Western blot analysis (Fig. 6). However, although mutant L77R HBsAg was very well colocalized with the Golgi 58K protein (Fig. 9, bottom), the wild-type HBsAg was not restricted to the Golgi compartment. Instead, it distributed throughout the entire cytoplasm (Fig. 9, top). In Fig. 9j to l, the Golgi compartment (green) was enlarged to a size similar to that of the nuclei, and the HBsAg (red) formed a letter “c” shape with the opening facing the nucleus. This kind of c-shaped staining pattern was absent in pECE24-transfected cells and was present in at least 25% of L77R-transfected cells, independent of their Golgi sizes (data not shown). The absence of HBsAg in the Golgi compartment immediately adjacent to the nucleus in Fig. 9l was reminiscent of the integrin (green)-containing Golgi compartments without HBsAg (red) in Fig. 7l and one cell near the left border in Fig. 7i. In summary, mutant L77R HBsAg almost always accumulated in ER-Golgi, independent from the quantity of HBsAg in those compartments. In contrast, wild-type pECE24, mutant W74L, and mutant W74L/L77R HBsAg tended to be distributed throughout the cytoplasm outside the ER-Golgi.

Lack of detectable ER stress response in cells transfected with mutant L77R small S envelope protein. The accumulation of the L77R HBsAg in ER-Golgi in transfected Huh7 cells predicts the induction of ER stress response. To test this prediction, we compared the Grp78 protein and RNA levels in nontransfected Huh7 cells and cells transfected with wild-type pECE24 or mutant L77R envelope expression vectors (Fig. 10). To our surprise, we detected neither increase of Grp78 mRNA by Northern blot analysis (Fig. 10a) nor of Grp78 proteins by Western blot analysis (Fig. 10b). In contrast, upon treatment with tunicamycin (an ER stress inducer),

FIG. 9. Accumulation of mutant L77R HBsAg in Golgi as visualized by immunofluorescence microscopy. Huh7 cells were transiently cotransfected as described in the legends to Fig. 7 and 8. After fixation, cells were stained for HBsAg (red), and a Golgi-specific marker, G58K (green). (Top) HBsAg of wild-type pECE24-transfected cells displayed a diffuse granular staining throughout the entire cytoplasm and did not colocalize with the Golgi. In fact, the staining intensity of wild-type HBsAg often became highly reduced at the Golgi compartment (data not shown). (Bottom) Mutant L77R HBsAg (red) colocalized very well with the Golgi marker G58K protein (green). (j and l) The Golgi compartment has a size similar to the nuclei, and the HBsAg formed a letter “c” shape with the opening facing the nucleus. This kind of c-shaped staining pattern was prevalent in transfected cells expressing L77R HBsAg (data not shown). The blue color of the nucleus was stained with DAPI. Scale bar, 10 μ m.

Grp78-specific mRNA or protein levels increased by approximately fourfold in both transfected and nontransfected Huh7 cells (Fig. 10a to c). The tubulin protein was included as a control for sample loadings (Fig. 10b and c, bottom). Consistent with the Western blot results, an immunofluorescence assay of pECE24 or L77R transfected and nontransfected control cells showed no increase in the amounts of Grp78 proteins (data not shown). Finally, tunicamycin treatment has been reported to activate the HBV pre-S2/S promoter in Huh7 cells by about 20 fold (46). Consistent with the lack of increased expression of Grp78 in L77R-transfected Huh7 cells (Fig. 10a and b), we detected no increase of the pre-S2/S mRNA in

tunicamycin-treated Huh7 cells by Northern blot analysis (data not shown).

DISCUSSION

Lack of immature secretion phenotype in vivo. The immature secreted virions may have a selective disadvantage in vivo (e.g., less infectious), leading to the emergence of compensatory mutations, such as core mutants P5T and P130T (11, 49). The possible existence of unknown compensatory mutations could explain the lack of abundant immature genomes in serum samples 9, 17, and 18, despite the presence of 97L muta-

FIG. 10. Accumulation of mutant L77R small S envelope protein in ER-Golgi of Huh7 cells does not induce detectable ER stress response. (a) Total RNAs were isolated from Huh7 cells cotransfected with pECE24 or L77R with F97L SK/O, treated or untreated with tunicamycin (50 μ g/ml) for 16 h. Northern blot analysis showed no apparent difference in the 2.7-kb Grp78-specific mRNA levels between nontransfected control cells and cells transfected with pECE24 or L77R (top). However, when cells were treated with tunicamycin, Grp78 mRNA levels increased by approximately fourfold in both transfected and nontransfected control cells (top). The 18S and 28S ribosomal RNAs from the cell lysates of the transfected culture were visualized by ethidium bromide staining on the agarose gel and served as an internal control for the RNA sample loadings (bottom). An RNA ladder was included as a size marker (data not shown). (b) Western blot analysis of Huh7 cells cotransfected with pECE24 or L77R with F97L SK/O showed no apparent increase in the amounts of Grp78 protein compared to the nontransfected control. When nontransfected control cells were treated with tunicamycin (Tuni.), Grp78 protein levels increased by approximately fourfold (top). The tubulin protein shown in the bottom panel served as an internal control for the amounts of total proteins loaded in each lane (bottom). (c) Western blot analysis of tunicamycin-treated Huh7 cells, which were cotransfected with pECE24 or L77R and F97L SK/O, showed a fourfold increase in the amounts of Grp78 protein compared to nontransfected and untreated control cells (top). The tubulin protein in the lower panel served as an internal control for the amounts of total proteins loaded in each lane (bottom).

tions (Fig. 1 and Table 1). Alternatively, the physiological status of host hepatocytes infected with HBV 97L variants could play a role in influencing the phenotypic expression of mutant 97L. For example, we found that the immature secretion phenotype of mutant 97L was more pronounced in Huh7 than in HepG2 cells (48).

A rare small S envelope mutation L77R is necessary and sufficient for the low-level secretion of virions and HBsAg. In our search for compensatory mutations in the HBV envelope genes of sample 17, we identified two small S envelope mutations, W74L and L77R, within the putative core-envelope interaction region. Because of the overlapping nature of the open reading frames shared by the L, M, and S envelope genes, the W74L and L77R mutations are present in all three envelope proteins. Based on complementation experiments between plasmid F97L SK/O and mutant L77R small S envelope expression vector (Fig. 3), the L77R small S envelope protein is necessary and sufficient for the pleiotropic phenotypes, including low-level secretion of virions (Fig. 3a) and HBsAg (Fig. 4a), low-level intracellular HBsAg by ELISA (Fig. 4b), increased accumulation of intracellular HBsAg in ER and Golgi (Fig. 7 to 9), and full-length RC from replicative intermediates (Fig. 3b).

A rare small S envelope mutant W74L/L77R is wild-type like in secretion of virions and HBsAg. We surveyed a total of 216 envelope sequences in GenBank and found only 1 sequence with the L77R mutation (accession number S41871), only 1 with a W74G mutation (accession number AAL30461), no W74L single mutation, and no W74L/L77R double mutations. This indicates that both W74L and L77R mutations are very rare. The rare occurrence of mutant L77R is consistent with its selective disadvantage of reduced virion secretion. The W74L mutation can suppress the phenotype of L77R, and thus the double mutant W74L/L77R is wild-type like in terms of the secretion of HBsAg and virions (Fig. 3 and 4) and the subcellular distribution of HBsAg (Fig. 7). The chances of fortuitously creating a perfect compensatory mutation during in vitro PCR in four out of four bacterial clones are extremely low.

Relationship between low-level virion secretion and suppression of immature secretion. Although both pre-S1 mutant A119F and core mutant P5T have a low-level virion secretion phenotype and both are compensatory for the core mutant I97L (11, 27), a low-level virion secretion phenotype is neither necessary nor sufficient for the suppression of the immature secretion from core mutant I97L. For example, the core mutant P130T has no low-level virion secretion, yet it is still compensatory for mutant 97L (49). Conversely, the small S envelope mutant L77R and the preS1 mutant L112F have a low-level virion secretion phenotype, yet they are not compensatory for the immature secretion of core mutant 97L (Fig. 3) (27).

Positive or negative correlation between intracellular HBsAg and virion secretion? It is known that both L and S envelope proteins are required for HBV virion secretion (4, 30, 43). Therefore, it is conceivable that a reduced intracellular level of HBsAg of mutant L77R (Fig. 4b) could result in a low level of secreted virions (Fig. 3a). On the other hand, we speculate that the >10-fold-reduced virion secretion of mutant L77R (Fig. 3a) is not entirely due to the 2-fold-reduced intracellular level of soluble HBsAg (Fig. 4b). Rather, the aberrant core-envelope interaction of mutant L77R probably contrib-

utes to the reduced efficiency of envelopment and assembly, leading to reduced virion release. This interpretation is consistent with the immunofluorescence data (Fig. 7 to 9) and the preferential accumulation of full-length RC-form DNA in cells transfected with mutant L77R and F97L SK/O (Fig. 3b).

Artificially engineered mutations within the putative core-envelope interaction loop of the small S envelope protein. Previously, Löffler-Mary et al. focused on the three arginine residues (R73, R78, and R79) in the core-envelope interaction domain of the small S envelope (29). They reported that replacement of these arginines by uncharged residues completely blocked HBsAg release. Taken together, our current studies of mutant L77R lend further support to a critical role of the putative core-envelope interaction domain for the successful release of virions and HBsAg. Also, the replacement of leucine-77 by several other amino acids does not result in low-level secretion (S. Le Pogam and C. Shih, unpublished results).

Low-level virion secretions caused by mutations near or within the group *a* determinant. The small S envelope protein contains a so-called group *a* determinant, which consists of neutralizing epitopes spanning a region around aa 124 to 147 (Fig. 2). A G119E mutant and a G145R mutant around this group *a* determinant have been reported to have low-level secretions of both virions and HBsAg (22, 23). Kalinina et al. reported that the G145R mutation had no effect on the intracellular level of L and S envelope proteins by Western blot analysis; however, the ELISA signals of secreted HBsAg in cell culture medium of mutant G145R was reduced by 40% (22), a result similar to that with our L77R mutant. Unlike mutant G145R, mutant L77R exhibited reduced intracellular levels of HBsAg by ELISA (Fig. 4b) and increased intracellular levels of HBsAg by Western blot analysis (Fig. 6a to c). We found no G119E and G145R mutations in sample 17 and no compensatory effect of mutation G145R on the immature secretion phenotype of core mutant F97L (data not shown).

Relationship between low-level extracellular HBsAg and low-level virion secretion. At least four different naturally occurring small S envelope mutants (L77R, G145R, G119E, and R169P) simultaneously exhibited reduced levels of both extracellular HBsAg and virion secretion (Fig. 3a and 4a) (22, 23). This apparent correlation between reduced extracellular HBsAg and virions suggests that the secretion pathways of HBsAg subviral particles and virion particles may share some steps in common. However, in our previous studies, core mutants P5T, P5A, P5S, and L60V had normal levels of secreted HBsAg, despite the fact that they all had a low-level virion secretion phenotype (26). Similarly, an engineered R79K small S envelope mutant can support HBsAg secretion but not virion secretion (29). Taken together, secretions of HBsAg and virions appeared to be dissociable, suggesting that virion and subviral particles could be released via different secretory pathways. Alternatively, a more plausible explanation of these phenomena is that the envelopment efficiencies of these low-level virion secretion core mutants were reduced.

Pathology and subcellular accumulation of mutant small S envelope protein. Previously, HBsAg of HBV variants 9a and 9b, which contained multiple mutations, colocalized with calnexin in ER but not with the G58K protein in Golgi (21). In contrast, in our L77R small S protein, it colocalized with both

calnexin (Fig. 8) and G58K protein (Fig. 9). Therefore, unlike variants 9a and 9b, the reduced secretion of virion or HBsAg in mutant L77R is not simply due to an arrest at the step from ER to Golgi. It has been reported that an internally deleted pre-S1 mutant accumulated HBsAg in dilated perinuclear vesicles in tissue culture. This phenomenon was postulated to be a contributing factor in the pathogenesis of ground glass cells (45). Furthermore, the retention of HBsAg in distended ER has also been observed with a transgenic mouse overexpressing the L envelope protein (9). Upon treatment with lipopolysaccharide or gamma interferon, transgenic mice developed severe acute liver disease, while nontransgenic littermates were totally resistant (18). The fact that mutant L77R also appeared to cause HBsAg retention in the ER-Golgi raised an issue of whether this variant envelope protein could predispose the host hepatocytes to liver injury. This may explain the emergence of the W74L compensatory mutation in association with the L77R mutation in patient 17.

Lack of detectable induction of ER stress in Huh7 cells with accumulation of L77R HBsAg in ER-Golgi. Accumulation of an internally deleted large envelope protein in ER-Golgi can result in ER stress in Huh7 cells (46). However, our results (Fig. 10) detected no significant ER stress response, as measured by the Grp78 mRNA and protein levels in L77R-transfected Huh7 cells. Similarly, we detected no apparent increase in Grp94 protein levels in pECE24- and L77R-transfected Huh7 cells by Western blot analysis (data not shown). In contrast, tunicamycin-treated Huh7 cells exhibited approximately fourfold-increased levels of Grp78 mRNA and protein, respectively (Fig. 10). Perhaps the almost exclusive accumulation of the L77R small envelope protein in ER-Golgi is not as potent an ER stress inducer as that of the large envelope protein (46). Alternatively, since only approximately 50% of the ER proteins are bound by Grp78 (25), it is possible that the small envelope protein is not recognized by Grp78.

Lack of up-regulation of the pre-S2/S promoter by ER stress. Previously, an approximately 10-fold increase in pre-S2/S promoter activity of an *adw2* HBsAg subtype HBV was observed when the ER stress response was induced by the accumulation of the large envelope protein in ER-Golgi of Huh7 cells. A feedback loop for the coordinated synthesis of the small and large envelope proteins was proposed to explain this phenomenon (46). In contrast, we detected no induction of the pre-S2/S mRNA by Northern blot analysis when L77R or pECE24 transfected cells were later treated with tunicamycin, an ER stress inducer (data not shown). Stimulation of the pre-S2/S promoter by ER stress was believed to be mediated via the CCAAT box in the pre-S2/S promoter (46). The CCAAT box in the pre-S2/S promoter is highly conserved among different HBV genotypes, including the *ayw* HBsAg subtype used in this study. Whether ER stress can induce the pre-S2/S mRNA production remains to be further investigated in the future.

In summary, we identified and characterized two closely associated naturally occurring mutations (W74L and L77R) in a putative cytosolic loop of the HBV small S envelope protein. This cytosolic loop is believed to be important for HBV capsids to bind to the envelope proteins during virion morphogenesis. One unexpected feature of the L77R mutant S envelope protein is its accumulation in the ER and Golgi, probably due to

protein misfolding or aggregation. Immunofluorescence microscopy confirmed the accumulation of the L77R protein in the Golgi and probably induced the enrichment of the Golgi marker 58K protein. Even though the L77R mutant HBsAg is accumulated in the ER-Golgi, we did not detect any ER stress response by measuring the expression level of Grp78. This phenotype of mutant L77R can be pronounced or suppressed, depending on the absence or presence of another suppressor mutation W74L. Finally, retention of HBsAg in ER in transgenic mice has been shown to cause hepatocytes hypersensitive to inflammatory cytokines. It remains to be investigated in the future if the L-envelope protein-independent accumulation of HBsAg in ER-Golgi in mutant L77R transfected hepatocytes also results in any hypersensitivity to gamma interferon or tumor necrosis factor alpha. At present, it remains unclear if the L77R mutation has any effect on the L and M envelope proteins.

ACKNOWLEDGMENTS

P.K.C. is a McLaughlin Postdoctoral Fellow. F.-M.S. was supported in part by Taipei Medical University Hospital and Juei-Low Sung's Research Foundation, Taiwan. This work was supported by the John Sealy Foundation and NIH grants R01 CA70336 and CA84217 to C.S.

We thank James Ou for plasmid pECE24, Kathleen O'Connor for anti-integrin antibody, Jiaren Sun for anti-transferrin antibody, Robert Lanford for anti-core antibody, Amy S. Lee for advice on ER stress, Eugene Knutson and Tom Albrecht at the Optical Imaging Center of UTMB for their help in confocal laser scanning microscopy, and Margaret Newman in C. Shih's laboratory for careful reading of the manuscript.

REFERENCES

- Beames, B., and R. E. Lanford. 1993. Carboxy-terminal truncations of the HBV core protein affect capsid formation and the apparent size of encapsidated HBV RNA. *Virology* **194**:597-607.
- Beasley, R. P., L. Y. Hwang, C. C. Lin, and C. S. Chien. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* **ii**:1129-1133.
- Bozkaya, H., B. Ayola, and A. S. Lok. 1996. High rate of mutations in the hepatitis B core gene during the immune clearance phase of chronic hepatitis B virus infection. *Hepatology* **24**:32-37.
- Bruss, V., and D. Ganem. 1991. The role of envelope proteins in hepatitis B virus assembly. *Proc. Natl. Acad. Sci. USA* **88**:1059-1063.
- 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.
- Bruss, V. 1997. A short linear sequence in the pre-S domain of the large hepatitis B virus envelope protein required for virion formation. *J. Virol.* **71**:9350-9357.
- Chang, S. F., H. J. Netter, M. Bruns, R. Schneider, K. Frolich, and H. Will. 1999. A new avian hepadnavirus infecting snow geese (*Anser caerulescens*) produces a significant fraction of virions containing single-stranded DNA. *Virology* **262**:39-54.
- Chisari, F. V., P. Filippi, A. McLachlan, D. R. Milich, M. Riggs, S. Lee, R. Palmiter, R. D., Pinkert, C. A., 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.
- Chisari, F. V., P. Filippi, J. Buras, A. McLachlan, H. Popper, C. A. Pinkert, R. D. Palmiter, and R. L. Brinster. 1987. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. *Proc. Natl. Acad. Sci. USA* **84**:6909-6913.
- Chisari, F. V., and C. Ferrari. 1995. Hepatitis B virus immunopathogenesis. *Annu. Rev. Immunol.* **13**:29-60.
- Chua, P. K., Y. M. Wen, and C. Shih. 2003. Coexistence of two distinct secretion mutations (P5T and I97L) in hepatitis B virus core produces a wild-type pattern of secretion. *J. Virol.* **77**:7673-7676.
- Chuang, W. L., M. Omata, T. Ehata, O. Yokosuka, Y. Ito, F. Imazeki, S. N. Lu, W. Y. Chang, and M. Ohto. 1993. Precore mutations and core clustering mutations in chronic hepatitis B virus infection. *Gastroenterology* **104**:263-271.
- Chuang, W. L., M. Omata, T. Ehata, O. Yokosuka, and M. Ohto. 1993. Concentrating missense mutations in core gene of hepatitis B virus. Evidence for adaptive mutation in chronic hepatitis B virus infection. *Dig. Dis. Sci.* **38**:594-600.
- Ehata, T., M. Omata, O. Yokosuka, K. Hosoda, and M. Ohto. 1992. Variations in codons 84-101 in the core nucleotide sequence correlate with hepatocellular injury in chronic hepatitis B virus infection. *J. Clin. Investig.* **89**:332-338.
- Ehata, T., M. Omata, W. L. Chuang, O. Yokosuka, Y. Ito, K. Hosoda, and M. Ohto. 1993. Mutations in core nucleotide sequence of hepatitis B virus correlate with fulminant and severe hepatitis. *J. Clin. Investig.* **91**:1206-1213.
- Ganem, D., and R. Schneider. 2001. *Hepadnaviridae*: the viruses and their replication, p. 2923-2970. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), *Fields virology*, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Gerelsaikhan, T., J. E. Tavis, and V. Bruss. 1996. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J. Virol.* **70**:4269-4274.
- Gilles, P. N., D. L. Guerrette, R. J. Ulevitch, R. D. Schreiber, and F. V. Chisari. 1992. HBsAg retention sensitizes the hepatocyte to injury by physiological concentrations of interferon-gamma. *Hepatology* **16**:655-663.
- Gunther, S., L. Fischer, I. Pult, M. Sterneck, N. Piwon, and H. Will. 1999. Naturally occurring variants of hepatitis B virus. *Adv. Virus Res.* **52**:25-137.
- Hosono, S., P. C. Tai, W. Wang, M. Ambrose, D. G. Hwang, T. T. Yuan, B. H. Peng, C. S. Yang, C. S. Lee, and C. Shih. 1995. Core antigen mutations of human hepatitis B virus in hepatomas accumulate in MHC class II-restricted T cell epitopes. *Virology* **212**:151-162.
- Kalinina, T., A. Riu, L. Fischer, H. Will, M. Sterneck. 2001. A dominant hepatitis B virus population defective in virus secretion because of several S-gene mutations from a patient with fulminant hepatitis. *Hepatology* **34**:385-394.
- Kalinina, T., A. Iwanski, H. Will, and M. Sterneck. 2003. Deficiency in virion secretion and decreased stability of the hepatitis B virus immune escape mutant G145R. *Hepatology* **38**:1274-1281.
- Khan, N., M. Guarnieri, S. H. Ahn, J. Li, Y. Zhou, G. Bang, K. M. Kim, J. R. Wands, and S. Tong. 2004. Modulation of hepatitis B virus secretion by naturally occurring mutations in the S gene. *J. Virol.* **78**:3262-3270.
- Koschel, M., D. Oed, T. Gerelsaikhan, R. Thomssen, and V. Bruss. 2000. Hepatitis B virus core gene mutations which block nucleocapsid envelopment. *J. Virol.* **74**:1-7.
- Lee, A. S. 2005. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* **35**:373-381.
- Le Pogam, S., T. T. Yuan, G. K. Sahu, S. Chatterjee, and C. Shih. 2000. Low-level secretion of human hepatitis B virus virions caused by two independent, naturally occurring mutations (P5T and L60V) in the capsid protein. *J. Virol.* **74**:9099-9105.
- Le Pogam, S., and C. Shih. 2002. Influence of a putative intermolecular interaction between core and the pre-S1 domain of the large envelope protein on hepatitis B virus secretion. *J. Virol.* **76**:6510-6517.
- Le Seyec, J., P. Chouteau, I. Cannie, C. Guguen-Guillouzo, and P. Gripon. 1998. Role of the pre-S2 domain of the large envelope protein in hepatitis B virus assembly and infectivity. *J. Virol.* **72**:5573-5578.
- Loffler-Mary, H., J. Dumortier, C. Klentsch-Zimmer, and R. Prange. 2000. Hepatitis B virus assembly is sensitive to changes in the cytosolic S loop of the envelope proteins. *Virology* **270**:358-367.
- Melegari, M., P. P. Scaglioni, and J. R. Wands. 1997. The small envelope protein is required for secretion of a naturally occurring hepatitis B virus mutant with pre-S1 deleted. *J. Virol.* **71**:5449-5454.
- Molnar-Kimber, K. L., V. Jarocki-Witek, S. K. Dheer, S. K. Vernon, A. J. Conley, A. R. Davis, and P. P. Hung. 1988. Distinctive properties of the hepatitis B virus envelope proteins. *J. Virol.* **62**:407-416.
- Ou, J. H., and W. J. Rutter. 1987. Regulation of secretion of the hepatitis B virus major surface antigen by the pre-S1 protein. *J. Virol.* **61**:782-786.
- Perlman, D., and J. Hu. 2003. Duck hepatitis B virus virion secretion requires a double-stranded DNA genome. *J. Virol.* **77**:2287-2294.
- Persing, D. H., H. E. Varmus, and D. Ganem. 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* **234**:1388-1391.
- Ponsel, D., and V. Bruss. 2003. Mapping of amino acid side chains on the surface of hepatitis B virus capsids required for envelopment and virion formation. *J. Virol.* **77**:416-422.
- Shih, C., P. C. Tai, W. Whitehead, S. Hosono, C. S. Lee, and C. S. Yang. 1996. Hepatitis B and C viruses and liver cancer, p. 824-834. In J. R. Bertino (ed.), *Encyclopedia of cancer*, vol. II. Academic Press, Inc., London, United Kingdom.
- Shih, C. 2003. Functional significance of naturally occurring hepatitis B virus variants, p. 23-41. In S. Locarnini and C. L. Lai (ed.), *Human virus guides—human hepatitis B viruses*. International Medical Press (IMP), London, United Kingdom.
- Stirk, H. J., J. M. Thornton, and C. R. Howard. 1992. A topological model for hepatitis B surface antigen. *Intervirology* **33**:148-158.
- Suk, F. M., M. H. Lin, M. Newman, S. Pan, S. H. Chen, J. D. Liu, and C. Shih. 2002. Replication advantage and host factor-independent phenotypes attributable to a common naturally occurring capsid mutation (I97L) in human hepatitis B virus. *J. Virol.* **76**:12069-12077.
- Summers, J., and W. S. Mason. 1982. Replication of the genome of a

- hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* **29**:403–415.
41. **Tai, P. C., D. Banik, G. I. Lin, S. Pai, K. Pai, M. H. Lin, G. Yuoh, S. Che, S. H. Hsu, T. C. Chen, T. T. Kuo, C. S. Lee, C. S. Yang, and C. Shih.** 1997. Novel and frequent mutations of hepatitis B virus coincide with a major histocompatibility complex class I-restricted T-cell epitope of the surface antigen. *J. Virol.* **71**:4852–4856.
42. **Tencza, M. G., and J. E. Newbold.** 1997. Heterogeneous response for a mammalian hepadnavirus infection to acyclovir: drug-arrested intermediates of minus-strand viral DNA synthesis are enveloped and secreted from infected cells as virion-like particles. *J. Med. Virol.* **51**:6–16.
43. **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.
44. **Wei, Y., J. E. Tavis, and D. Ganem.** 1996. Relationship between viral DNA synthesis and virion envelopment in hepatitis B viruses. *J. Virol.* **70**: 6455–6458.
45. **Xu, Z., and T. S. Yen.** 1996. Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. *J. Virol.* **70**:133–140.
46. **Xu, Z., G. Jensen, and T. S. Yen.** 1997. Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum. *J. Virol.* **71**:7387–7392.
47. **Yuan, T. T., G. K. Sahu, W. E. Whitehead, R. Greenberg, and C. Shih.** 1999. The mechanism of an immature secretion phenotype of a highly frequent naturally occurring missense mutation at codon 97 of human hepatitis B virus core antigen. *J. Virol.* **73**:5731–5740.
48. **Yuan, T. T., P. C. Tai, and C. Shih.** 1999. Subtype-independent immature secretion and subtype-dependent replication deficiency of a highly frequent, naturally occurring mutation of human hepatitis B virus core antigen. *J. Virol.* **73**:10122–10128.
49. **Yuan, T. T., and C. Shih, C.** 2000. A frequent, naturally occurring mutation (P130T) of human hepatitis B virus core antigen is compensatory for immature secretion phenotype of another frequent variant (I97L). *J. Virol.* **74**: 4929–4932.