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The Small Envelope Protein Is Required for Secretion of a Naturally Occurring Hepatitis B Virus Mutant with Pre-S1 Deleted

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Naturally occurring deletions in the hepatitis B virus pre-S1 domain have been frequently found during persistent viral infection. In this study we have investigated the functional properties of a mutant viral genome that carries an in-frame deletion of 183 nucleotides in the pre-S1 region. This deletion removes the promoter of the small envelope gene. Transfection into human hepatocellular carcinoma cells of a replication-competent construct containing this deletion resulted in an increase of intermediate DNA replicative forms compared to those produced by wild-type hepatitis B virus. Northern blot analysis revealed that such cells lack the 2.1-kb transcripts encoding the small envelope protein and that hepatitis B surface antigen was absent as well. Furthermore, nucleocapsids containing the genome with pre-S1 deleted were not secreted, and the deleted large envelope protein was retained with the cytoplasm and exhibited a perinuclear pattern of distribution. However, coexpression with the small envelope protein was sufficient to restore virion secretion and to change the cellular distribution of the deleted large envelope protein. In addition, the creation of point mutations that prevent the synthesis of large or small envelope proteins also inhibited viral secretion and led to increased levels of hepatitis B virus intermediate replicative forms within the cell. These studies suggest that naturally occurring viral mutants with pre-S1 deletions involving the promoter region of the small envelope gene will generate a deleted large envelope protein that is retained in the endoplasmic reticulum, resulting in the accumulation of nucleocapsids containing viral DNA; transcomplementation with the wild-type small envelope protein will allow mutant virion secretion to occur.

Mutations and deletions in the hepatitis B virus (HBV) genome have frequently been found during persistent viral infection. The most important recently identified mutational "hot spots" of the viral genome include the precore and core promoter regions, the core and precore open reading frames (ORFs), and the pre-S region. The molecular mechanism(s) that allows for the emergence of these mutant viral strains has not been established. Thus, investigations into the generation of such viral variants may lead to an understanding of virus-host relationships and to the cellular events involved in viral persistence (18). For example, it is likely that mutations of the viral genome that confer a high-replication phenotype may eventually allow the variant strains to predominate over the wild-type virus providing that they are secreted from the cell and remain infectious. The relationship between the biological properties of HBV mutants and the clinical course of disease needs to be established. Therefore, it is important to study the functional properties of these variant viral strains in order to determine if they display a different biological behavior that may either contribute to viral latency (2) or produce severe and progressive liver disease.

In the present investigation, the characteristics of a naturally occurring viral variant with pre-S1 deleted were explored. This mutant viral strain has been frequently found in sera of individuals with chronic hepatitis and cirrhosis (9, 15, 16, 26, 28). In this study, we assessed (i) the intracellular level of DNA replicative intermediate forms of the genome with pre-S1 deleted compared to that of wild-type HBV and (ii) the type(s) of envelope proteins required to promote virion secretion from the cell. The findings demonstrate that a large (L) envelope protein with a deleted pre-S1 region will export the core particles only if the small (s) protein is also provided in *trans* by wild-type virus. The biological implication of these findings is that the genomes with pre-S1 deleted will accumulate within hepatocytes and may contribute to cellular injury.

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MATERIALS AND METHODS

Analysis of serum samples. Extraction of DNA from serum as well as PCR amplification and the subsequent cloning and sequencing of amplified HBV DNA was performed as previously described (15).

Plasmids. The replication-competent viral vectors used in this study were derived from a wild-type payw1.2 HBV construct more than one genome in length (D genotype [17]) (25). This plasmid will express the 3.5-kb pregenomic RNA from the endogenous core promoter. The following expression vectors were also prepared as depicted in Fig. 1. In brief, the pΔ183 construct was made by the insertion of the *Bst*EII (nucleotide [nt] 2813)-*Avr*II (nt 176) fragment carrying a 183-nt deletion into the wild-type payw1.2 genome following partial digestion with the *Bst*EII and *Avr*II restriction enzymes. Additional constructs were produced by site-directed mutagenesis (pAlter system; Promega Co., Madison, Wis.) to change the ATG initiation codons for the pre-S1 and s genes into ACG, leaving unaltered the amino acid sequence of the overlapping polymerase protein. These constructs are designated payw L− and payw s−.

In addition, three constructs expressing HBV envelope proteins were generated for transcomplementation studies. One construct, designated penv+, transcribes the L, middle (M), and s proteins under the control of endogenous promoters as previously described (15). Another construct (pM+s+) allows for the synthesis of M and s envelope proteins under the control of the endogenous s promoter. This expression vector was prepared by digestion of the parental payw1.2 with *Apa*I [restriction site in the pGEM-7Zf(+) polylinker; Promega Co.] and *Bst*EII (nt 2813) restriction enzymes followed by gel purification of the resulting 5.7-kb DNA fragment. The staggered ends were blunted by the Klenow fragment of DNA polymerase I followed by self-religation with T4 DNA ligase. The final construct, designated pCMV s, produces the s envelope protein under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter and

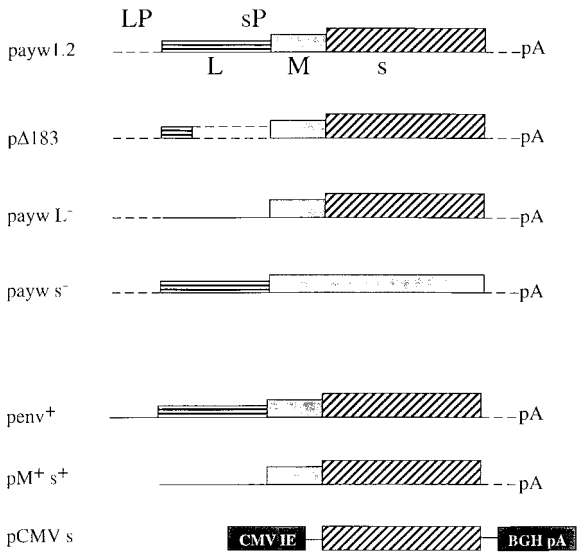

FIG. 1. Sketch illustrating the various expression constructs used in the study. The first four constructs represent HBV replication-competent vectors of more than a genome in length driven by the endogenous core promoter and endogenous polyadenylation site (pA). payw1.2 represents the vector that produces wild-type HBV of the D genotype. The pΔ183 construct carries the 183-nt deletion in the pre-S1 region and was inserted into the payw1.2 backbone. The payw L− and payw s− constructs differ from the wild-type virus only by missense mutations in the initiation codons that abolish the synthesis of L and s envelope proteins, respectively. The last three constructs express the L, M, and s envelope proteins under the control of the endogenous L envelope (LP) promoter (penv+); the M and s envelope proteins under the control of the s envelope (sP) promoter (pM+s+); and the s envelope protein expressed from the CMV IE promoter (pCMV s). This vector uses the bovine growth hormone pA (BGH pA).

was derived by deletion of the *Hin*dIII-*Eco*RI fragment followed by the filling in of the staggered ends by the Klenow fragment of DNA polymerase I and ligation of plasmid pCMV pre-S1. The latter contains the HBV sequence *Bgl*II (nt 2835)-*Fsp*I (nt 1798) inserted into the *Bam*HI-*Eco*RV sites of the pcDNA3 vector (Invitrogen, San Diego, Calif.) (Fig. 1).

Cell transfection studies. The HuH-7 and HepG2 hepatocellular carcinoma (HCC) cell lines were grown in Dulbecco's modified essential medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2–air mixture. Approximately 10^7 HCC cells were seeded into a 10-cm-diameter dish one day before transfection with plasmid DNA. Subsequently, 10 μg of the constructs that utilize the HBV endogenous promoter (i.e., payw1.2, pΔ183, payw L−, payw s−, penv+, and pM+s+) and 1 μg of construct pCMV s that utilizes the CMV IE promoter were transfected into HCC cells by the $CaPO_4$ technique ($CaPO_4$ transfection kit; 5′3′ Inc., Boulder, Colo.). In preliminary experiments, these vector amounts yielded similar expression of hepatitis B surface antigen (HBsAg) in the culture supernatants of transfected cells. The final concentration of plasmid DNA was kept constant by adding pGEM-7Zf(+) DNA. Cells and culture supernatants were harvested 2 and 5 days after transfection for RNA and DNA analysis, respectively. The transfection efficiency was controlled by the addition of 1 μg of pCMV Luc, and luciferase activity was subsequently measured in 1/100 of the cell lysate volume. This construct expresses the luciferase gene under the control of the CMV IE promoter and enhancer element. Transfection experiments were disregarded if the luciferase activity varied more than 20% within the cell lysates following transfection, and the loading of HBV DNA derived from core particles in the Southern blots was adjusted according to the luciferase activity. Another control for transfection efficiency was the concentration of hepatitis B e antigen (HBeAg) secreted into the cell culture supernatant as measured by the EBK kit (Incstar Co., Stillwater, Minn.) as well as HBsAg concentrations in the culture supernatants (30). The assay for the latter was performed in a linear range (0.5 to 50 ng/ml) on serial dilutions of culture medium. An excellent correlation was obtained between the luciferase activity in cell lysates and the levels of secreted viral antigens in cell culture supernatants in all transfection experiments. Finally, transfection studies were carried out at least five times with different plasmid preparations, and similar results were obtained.

Immunoprecipitation studies. Culture supernatants derived from transfected cells were cleared of cellular debris by centrifugation at 12,000 × *g* for 10 min and then loaded onto a 20% (wt/wt) sucrose-TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA) cushion followed by ultracentrifugation at 200,000 × *g* with a Beckman SW28 rotor at 15°C for 6 h. The pellet that contained viral particles as well as nonenveloped nucleocapsids was resuspended in 1 ml of TNE-protease inhibitor (Boehringer Mannheim Co., Indianapolis, Ind.)-containing buffer, and selective immunoprecipitation studies were performed at 4°C for 1 h with a MA18/7 monoclonal antibody (MAb) reacting with the N-terminal region of the L envelope protein (kind gift from W. H. Gerlich) (10) or with the 5C3 anti-hepatitis B surface (HBs) MAb that recognizes a conformational epitope on the major *a* determinant of HBsAg (30). Following this procedure, protein A or protein G Sepharose beads (Pharmacia Biotech Inc., Piscataway, N.J.), respectively, were added to the mixture at 4°C for 1 h and the immune complexes bound to the beads were subsequently isolated by centrifugation for 20 s. The Sepharose beads were washed five times with buffer containing TNE-protease inhibitors. The pellet was resuspended in TLB (100 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1% Nonidet P-40) solution containing magnesium acetate at a final concentration of 10 mM. A digestion with DNase I and RNase A was then performed for 30 min at 37°C to degrade contaminating plasmids, and HBV DNA from the immunoprecipitated virions was extracted as previously described (22). Immunoprecipitations for virion DNA performed with nonrelevant antibodies were negative.

Southern and Northern blot analysis. The HBV DNA derived from core particles and total cellular RNA were purified as previously described (25). Southern and Northern blot analysis was performed with randomly primed ^{32}P-labeled 3.2-kb HBV DNA or with a core-specific probe (*Bgl*II fragment, nt 1982 to 2421) as described previously (25).

Immunofluorescence studies. Transfected cells were grown on coverslips, washed two times in phosphate-buffered saline (PBS) at 4°C, and fixed with 95% ethanol–5% acetic acid (11). After a blocking reaction with 3% bovine serum albumin for 30 min at 37°C, the primary antibodies (mouse MAb MA18/7 or goat polyclonal anti-HBs antibody; DAKO Co., Carpinteria, Calif.) were added for 1 h in a humidified chamber at 37°C and the cells were washed three times with PBS. The second antibody conjugated to fluorescein isothiocyanate was added according to the manufacturer's instructions (Organon Teknika Co., Durham, N.C.), and the cells were examined under a Zeiss fluorescent microscope.

RESULTS

Characterization of virions containing a pre-S1 deletion. A virion containing a deletion between nt 2983 and 3167 in the pre-S1 ORF was discovered by PCR amplification of HBV DNA from the serum of an individual with chronic liver disease following alpha interferon therapy. This mutational event led to an in-frame deletion from amino acid (aa) 47 to 107 of the L envelope protein (15) of a D genotype HBV strain (17). As shown by the agarose gel fractionation of the PCR-amplified viral DNA, the deleted molecule, as represented by the lower band in the sample taken in January 1993 (Fig. 2A, lane 1.93), became the predominant viral species in serum during serial follow-up observations. However, note the presence of a small amount of full-length wild-type HBV DNA, as represented by the upper band in Fig. 2A. A similar high ratio of deleted-genome viral DNA to full-length wild-type viral DNA was evident when the PCR amplification was performed with oligonucleotides designed to amplify a fragment as large as 1.6 kb (data not shown). This experiment excluded the possibility that the predominance of the viral genome with the deletion was due to the preferred use of the smaller template as opposed to the full-length wild-type HBV DNA in the PCR amplification. In addition, another minor viral species with a 45-nt deletion was identified, and this mutation occurred within the boundaries of the major fragment with pre-S1 deleted. Subsequent sequence analysis and comparison to all known HBV strains in the database demonstrated that the 183-nt deletion occurred at consensus donor and acceptor splice sites as defined by the GU and AG nucleotides at the 5′ and 3′ regions of the deletion, respectively. It is noteworthy that the 183-nt deletion abolishes most transcription factor binding sites that map to the s promoter and that only the NF1 binding site remains (23). This deletion will also generate a mutant polymerase protein containing an in-frame deletion spanning aa 228 through 288 and located within the "spacer" region of the molecule (1).

Replication of the deleted viral genome. The viral genome with pre-S1 deleted was amplified from serum during the nat-

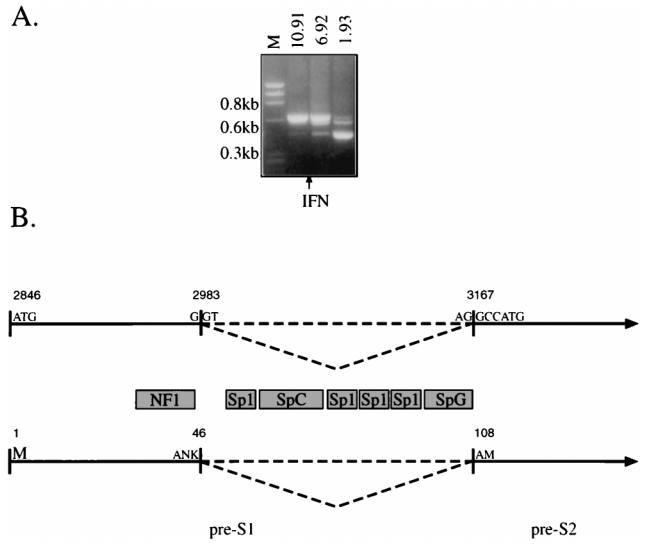

FIG. 2. (A) Agarose gel fractionation of PCR-amplified HBV DNA from the serum of a chronically infected individual who underwent alpha interferon therapy (IFN). Note the emergence and predominance of the HBV mutant with pre-S1 deleted over time. A viral species containing a smaller pre-S1 deletion (deletion of 45 nt) was also present. DNA molecular weight markers are on the left side. (B) Diagram of the HBV deleted region. The 183-nt deletion joins nt 2983 with 3167 in the pre-S1 ORF. The pre-S1 protein therefore has an aa 47 to 107 in-frame deletion. This deletion also overlaps the spacer region of the polymerase gene and results in an aa 228 to 288 in-frame deletion of the polymerase protein. Transcription factor binding sites (24) are lost in the s promoter, as depicted by the boxes. The only remaining transcription factor binding site is for NF1.

ural course of chronic hepatitis B virus infection. This finding implies that the pregenomic RNA with the deletion must have been efficiently packaged, reverse transcribed into DNA, and secreted from the infected hepatocyte. Therefore, we assessed the pattern of expression of the mutant HBV when transfected alone or in combination with vectors providing the envelope protein(s) in *trans*. Southern blot analysis of viral nucleocapsids derived from the cytoplasm revealed an increased level of replicative forms in cells that were transfected with the viral genome with the deletion compared to that in cells transfected with wild-type HBV (Fig. 3A; compare lanes 1 and 2). When pΔ183 was cotransfected with a plasmid that provided the L, M, and s proteins, the M and s proteins, or only the s protein (lanes 3 through 5), the level of deleted-DNA replicative forms became comparable to that found for wild-type virus (compare to lane 1).

Synthesis and secretion of envelope protein(s). The concentration of HBsAg was measured in culture supernatants following transfection of HuH-7 or HepG2 cells with the various envelope constructs. It was determined that cells transfected with pΔ183 alone did not produce HBsAg (Fig. 3B, lane 2). As expected, the concentrations of HBeAg secreted into the culture medium for the five constructs were similar.

The HBV RNA levels in HCC cells transfected with wild-type payw1.2 and pΔ183 constructs were examined. Total cellular RNA derived from HepG2 cells was hybridized with a 3.2-kb HBV probe, and transcripts of the expected size were found for the 3.5-kb pregenomic and 2.4- and 0.7-kb subgenomic RNA species. However, the 2.1-kb pre-S2/s gene transcripts were selectively missing from cells transfected with the pΔ183 construct (Fig. 4). When the same viral RNA was hybridized to a core-specific probe, a substantial proportion of the RNA species migrating at about 2.4 kb and containing the transcript for the L protein with the deletion was shown to also contain core-specific sequences. This finding is consistent with the presence of spliced core-s hybrid molecules, as previously described (6, 27).

Immunofluorescence studies. The immunofluorescence pattern obtained with MAb MA18/7 was characterized by a granular staining of the perinuclear region following transfection of HuH-7 cells with the pΔ183 construct (Fig. 5C). The staining pattern obtained with polyclonal anti-HBs antibodies yielded the same result, and this indicates that the staining was not due to reactivity of the pre-S1 MAb with degraded peptides and that the L envelope protein with the deletion was intact and contained the s envelope region (data not shown). Moreover, a Western blot analysis performed on the cell lysates derived from pΔ183-transfected cells revealed the physical integrity of the L protein with the deletion, as indicated by the presence of (i) a protein of the expected size reacting with an anti-pre-S1 MAb and (ii) multiple bands of higher molecular weight consistent with the hyperglycosylation forms of the L protein with the deletion (data not shown). The granular immunofluorescence staining pattern of transfected cells expressing the L protein with the deletion suggests retention in the endoplasmic

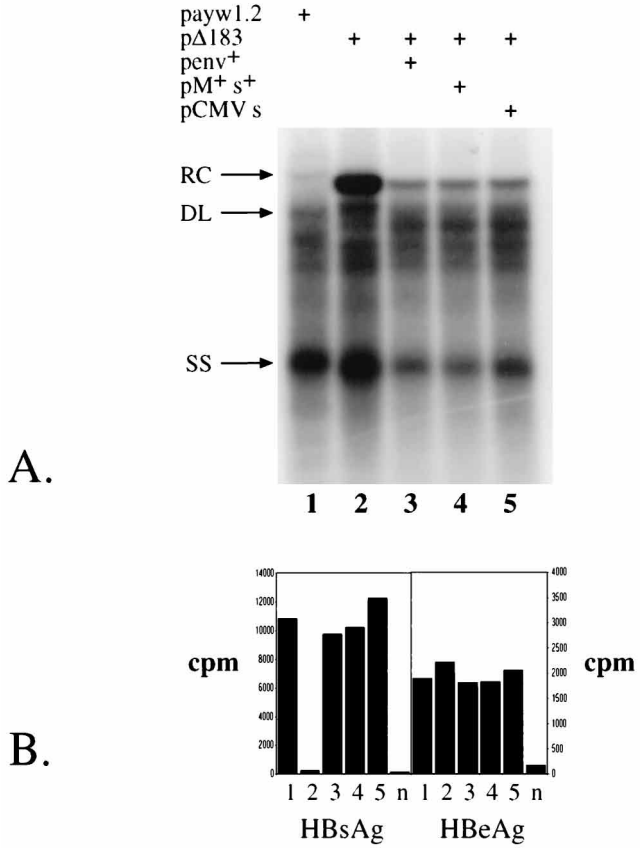

FIG. 3. (A) Southern blot analysis of HBV DNA derived from HepG2 cells transfected with wild-type payw1.2 (lane 1) or pΔ183 (lane 2). Cotransfection experiments were performed with pΔ183 and penv+ constructs that provide L, M, and s envelope proteins (lane 3), with a pM+s+ construct that provides M and s proteins (lane 4), or with a pCMV s construct that provides the s envelope proteins (lane 5) in *trans*. The relaxed circular (RC), double-strand linear (DL), and single-strand (SS) DNA species are indicated by the arrows on the left side. (B) HBsAg and HBeAg levels secreted into the medium of the above transfected cells as measured by radioimmunoassays. Note that cells transfected with pΔ183 do not secrete HBsAg. However, all constructs produce HBeAg at approximately equal concentrations in cell culture supernatants. n, mock-DNA-transfected cells.

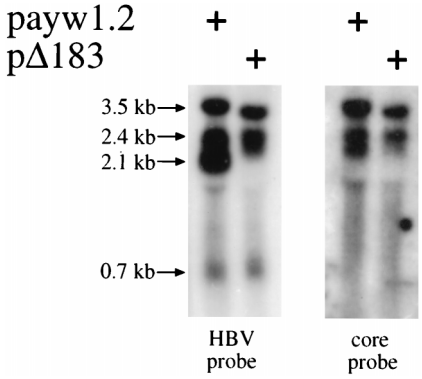

FIG. 4. Northern blot analysis of total cellular RNA extracted from HepG2 cells transfected with the payw1.2 and pΔ183 constructs. The left panel represents the 3.5-kb pregenomic RNA as well as 2.4- and 0.7-kb subgenomic species produced by hybridization with the 3.2-kb HBV DNA probe. Note that the RNA band corresponding to the 2.1-kb transcript size is missing in cells transfected with the pΔ183 construct. The right panel represents the result of the Northern blot analysis of the same RNA hybridized to a *Bgl*II fragment containing the HBV core gene. This probe recognizes 2.4-kb core-specific RNA transcripts in total cellular RNA extracted from cells transfected with both the payw1.2 and pΔ183 constructs.

reticulum (20, 31). In contrast, transfection of such cells with wild-type payw1.2 produced a pre-S1 staining reaction characterized by a fine and diffuse distribution of L envelope proteins throughout the cytoplasm (Fig. 5A). More important, a reversion to a wild-type cellular staining pattern was observed when pΔ183 was cotransfected with the pM+s+ and pCMV s expression vectors (Fig. 5E and G, respectively). These findings suggest that coexpression of s protein alone was sufficient to redistribute the L envelope protein with the deletion within the cytoplasm of transfected cells in a fashion similar to that of wild-type proteins.

Analysis of virion secretion. We then asked which envelope protein(s) was necessary to promote the secretion of nucleocapsids containing the genome with the deletion from the cell. Immunoprecipitation experiments were performed on the supernatants of transfected cells by using either 5C3 (anti-HBs) MAb and polyclonal anti-HBs antibodies or MAb MA18/7 recognizing the N terminus of pre-S1 to determine if pre-S1 epitopes with the deletion were present on the secreted virions. As shown in Fig. 6, viral DNA was absent from the supernatant of cells transfected with pΔ183 when anti-HBs or anti-preS1 MAbs were used to immunoprecipitate virions (lanes 2 and 6). However, cotransfection with penv+ that provided in *trans* all three wild-type envelope proteins restored virion secretion (lane 4). Viral DNA was also found in the supernatant when pΔ183 was cotransfected with a vector that provided only the s protein in *trans* (lanes 3 and 7). These findings suggest that the L protein with the deletion was present on the surface of the enveloped and secreted virions and was available for immunoprecipitation with the anti-HBs and pre-S1-specific MAbs (4, 5, 19, 21).

Replication of virions containing pre-S1 and s start codon mutations. Point mutations were produced in the initiation codons of the L and s envelope proteins in order to confirm that the increased levels of replicative intermediate forms derived from the viral genome with pre-S1 deleted were due to the impaired secretion of enveloped virions. These mutant species have been shown to be defective in particle secretion (3, 29). HCC cells transfected with the payw L− construct secreted HBsAg into the cell culture supernatant at levels comparable to that of wild-type payw1.2. As expected, HBsAg was undetectable in the supernatants derived from cells transfected with payw s− (data not shown). Southern blot analysis of core particles extracted from the cytoplasm of HCC cells transfected with payw s− and payw L− demonstrated increased levels of HBV replicative forms, as shown in Fig. 7, lanes 3 and 4, respectively. These levels of viral replication were comparable to those found in cells transfected with pΔ183 alone, as shown in lane 2. The levels of replicative forms exhibited by all three mutant genomes (i.e., pΔ183, payw s−, and payw L−) were higher than that observed with wild-type payw1.2, as shown in lane 1. Moreover, the level of HBV replicative forms accumulating in core particles was reduced to wild-type levels when payw s− was cotransfected with pCMV s

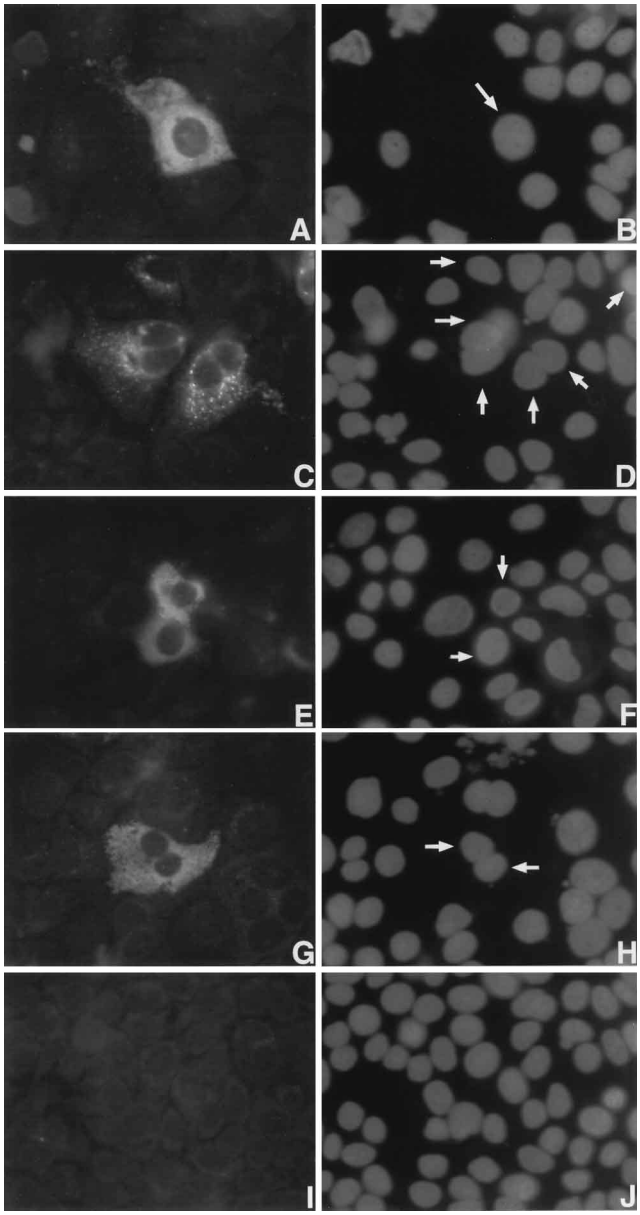

FIG. 5. Immunofluorescence studies of HuH-7 cells transfected with payw1.2 (A), pΔ183 (C), pΔ183 together with pM+s+ (E), and pΔ183 together with pCMV s (G) and of mock transfected cells (I) as revealed by the MA18/7 MAb. Nuclear staining, shown in panels B, D, F, H, and J, was performed with Hoechst dye 33258 (bisbenzimide). Transfected cells are marked by arrows.

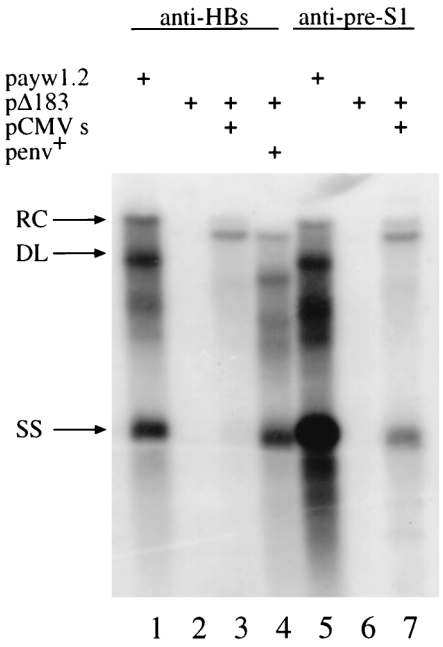

FIG. 6. Southern blot analysis of virions secreted into the cell culture supernatant from transfected HepG2 cells following immunoprecipitation with MAbs (see Materials and Methods). Cells transfected with the pΔ183 construct were unable to secrete enveloped virus as shown by lanes 2 and 6. In contrast, cotransfection with constructs expressing s, as shown in lanes 3 and 7, or with all three envelope proteins, as shown in lane 4, allowed for secretion of virions containing the genome with pre-S1 deleted into the culture medium. Note that the MA18/7 MAb immunoprecipitated virions from pΔ183-transfected cells only when the s envelope protein was provided in *trans*. For the definitions of RC, DL, and SS, see the legend for Fig. 3.

(lane 5) or when payw L− was cotransfected with penv+ (lane 6). We also observed that there was an increase in the relative ratio of relaxed circular DNA versus double-strand linear or single-strand DNA in cells transfected with pΔ183, payw s−, or payw L− (lanes 2, 3, and 4).

DISCUSSION

In the present investigation, the biological properties of an HBV mutant commonly found during persistent viral infection in various regions of the world were characterized. The viral genome with the deletion may have been generated by a splicing event since the boundary of the deletion contains consensus donor and acceptor splice sites that are conserved among all known HBV genotypes (17). This phenomenon may explain why this unique pre-S1 deletion has been found so often during persistent viral infection. Such a deletion removes all but one of the NF1 binding sites in the s promoter region. In transfected cells, the 2.1-kb transcripts are absent and there is a corresponding lack of HBsAg synthesis and secretion into the culture supernatant. In this regard, a previous study demonstrated that smaller deletions involving the most 5′ Sp1 and SpC binding sites (23) were sufficient to abolish HBsAg secretion following transfection into HCC cells (15).

By immunofluorescence studies, it was found that the lack of s envelope protein synthesis by the mutant genome appeared to induce retention of the L envelope protein with the deletion within the cytoplasm of transfected cells (12, 20, 31). However, coexpression with the s protein was sufficient to allow redistribution of the L envelope protein with the deletion, producing a more diffuse cytoplasmic staining pattern that is characteristic of the distribution of full-length wild-type L envelope protein. Immunoprecipitation experiments performed on the supernatants of transfected cells with anti-pre-S1 and anti-HBs MAbs demonstrated that the L envelope protein with the deletion alone was not capable of encapsidating mutant viral genomes and promoting their secretion from the cell. This finding has also been reported for the full-length L envelope protein when expressed by replication-competent vectors that do not express M and s proteins (3, 29). The striking accumulation of HBV DNA replicative intermediates in the cytoplasm of transfected cells is most likely due to the lack of s protein expression. Indeed, when the s protein was provided in *trans*, nucleocapsids containing the mutant viral genome were secreted from the cell.

The biological behavior of this L envelope protein with the deletion is consistent with the presence of (i) a retention signal in the amino terminus of the pre-S1 protein (12, 20, 31), (ii) a functional domain for interaction with the viral core particles (4, 5, 19, 21), and (iii) a location for the L envelope protein with the deletion on the coats of the viral particles (4, 5, 19, 21), as demonstrated by the immunoprecipitation experiments. It will be of interest to determine if additional small deletions in the remaining region of the L envelope protein will affect the cytoplasmic distribution and/or inhibit the process of virion morphogenesis and secretion from the cell.

In the duck hepatitis B virus (DHBV) model, mutations in the pre-S region of the p36 L envelope protein led to defects in covalently closed circular DNA (cccDNA) regulation (13). Such mutations were associated with the secretion of pre-S mutant virions capable of inducing cytopathic changes in infected cells (14). These experiments suggest that the DHBV wild-type L protein may promote persistent noncytopathic infection through a repression of viral replication. In our model

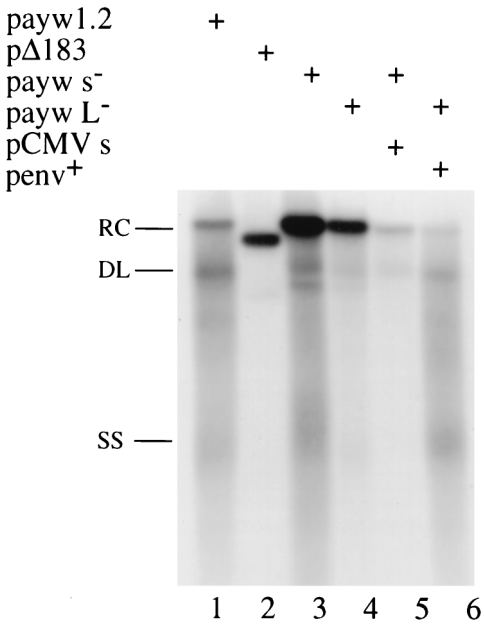

FIG. 7. Southern blot analysis of HBV DNA purified from nucleocapsids extracted from HepG2-transfected cells. Cells transfected with pΔ183 demonstrated an accumulation of HBV replicative forms (lane 2), similar to cells transfected with payw s− (lane 3) and payw L− (lane 4). Lane 1 represents levels of HBV replicative forms observed in cells transfected with wild-type payw1.2. However, when payw s− was cotransfected with pCMV s (lane 5) or payw L− was cotransfected with penv+ (lane 6), the accumulation of nucleocapsids containing HBV DNA replicative forms in the cytoplasm was reduced. For the definitions of RC, DL, and SS, see the legend for Fig. 3.

system, it was not possible to test whether the lack of secretion of the mutant virions resulted in an accumulation of cccDNA forms in the nucleus because HCC cells transfected with HBV DNA are not generally proficient in providing cccDNA templates for pregenomic RNA synthesis and subsequent DNA replication. However, the biological consequences of these events in vivo may be an increase in the cccDNA pool and, when associated with a block in viral secretion, the accumulation of mutant viral genomes, which may be cytotoxic to hepatocytes. For example, an increase in the processing of viral peptides in the context of HLA class I antigen presentation may activate cytotoxic T lymphocytes and promote liver injury (8). The mutant genomes with L envelope proteins carrying a deletion could contribute to the formation of ground glass hepatocytes (31), as has been observed in transgenic mice overexpressing the native L envelope protein (7). Additional investigations of such HBV deletion mutants will provide an understanding of host-virus interactions with respect to the induction of liver injury and the persistence of viral genomes within hepatocytes.

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M. Melegari and P. P. Scaglioni contributed equally to this work.

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