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Identification and Characterization of a Structural Protein of Hepatitis B Virus: A Polymerase and Surface Fusion Protein Encoded by a Spliced RNA

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The hepatitis B virus (HBV) genome is known to contain four conserved and overlapped open reading frames (ORFs) encoding the viral core, polymerase (P), surface (S), and X proteins. Whether HBV encodes other proteins has long been a major interest in the field. Using ^{32}P-labeling of an introduced protein kinase A site attached to the N- or C-terminus of the HBV polymerase gene, a 43-kDa P–S fusion protein was detected in cell lysate, secreted virions, and 22-nm subviral particles. Immunobiochemical studies showed that the 43-kDa protein contains the epitopes of the N-terminus of polymerase and most parts of the surface proteins. This 43-kDa protein was shown to be a glycoprotein, similar to the surface protein. RT-PCR and sequence analyses identified a spliced mRNA which was derived from pregenomic RNA with a deletion of 454 nucleotides (nt) from nt 2447 to 2902. This splice event creates a P–S fusion ORF. This finding is consistent with the result obtained from an immunobiochemical study. Mutations at the splice donor or acceptor site on the HBV genome abrogated the production of the 43-kDa protein. These mutants had no effect on viral replication in transfected HuH-7 cells. However, this P–S fusion protein is able to substitute for the LS protein in virion maturation. On the basis of these results, we conclude that the 43-kDa protein is a polymerase-surface fusion protein encoded by a spliced RNA. Similar to the LS protein, the 43-kDa P–S fusion protein is a structural protein of HBV and might play a role in the HBV life cycle.

INTRODUCTION

The hepatitis B virus (HBV) is a member of the *Hepadnaviridae,* a family of small enveloped viruses with partially double-stranded DNA genomes (for recent reviews see Ganem, 1996; Nassal and Schaller, 1993, 1996; and Nassal, 1996). HBV infection is a major health problem in the world, particularly in East Asia and Central Africa, where an estimated 10% of the population are HBV carriers and many of them die from liver cirrhosis and hepatocellular carcinoma. The HBV genome is approximately 3200 base pairs in length and consists of four overlapping open reading frames (ORFs) which encode the viral core, polymerase (P), surface (S), and X proteins (Fig. 1A). The three surface proteins (or surface antigens, HBsAg) are translated from a single *S* ORF via three different in-phase start codons (Fig. 1A). The small surface antigen (SS) contains 226 amino acids. The middle surface (MS) protein is extended by an additional N-terminal 55 amino acids of pre-S2 domain and the large surface (LS) protein by an additional 163 amino acids of pre-S2 plus pre-S1 domain (subtype ayw, Heermann *et al.,* 1984). Part of the surface proteins are N-glycosylated. Recent studies indicate an important relationship between the surface proteins and virion maturation or virion infectivity (Bruss *et al.,* 1994; Bruss and Thomssen, 1994; Heermann and Gerlich, 1991; Pontisso *et al.,* 1989; and Le Seyec *et al.,* 1998). However, with the exception of a few reports (Dyson and Murray, 1995; Poisson *et al.,* 1997), the interaction between nucleocapsids and the surface proteins is still poorly defined.

A unique feature of hepadnaviruses is their unusual mechanism of viral DNA replication involving reverse transcription of pregenomic RNA by viral encoded polymerase, a pivotal process similar to that of a retrovirus. However, there are also features unique to retroviruses that had not been discovered in HBV, such as: (i) all retroviruses utilize an RNA splicing mechanism to generate the *env* mRNA; (ii) some retroviruses, such as HIV-1, produce several spliced RNAs to encode regulatory proteins (Tat, Rev, Vif, Vpr, and Nef) which are either essential for viral replication or capable of modulating replication; (iii) some retroviral proteins are produced by posttranslational processing of polyprotein precursors (reviewed in reference to Vaishnav and Wong-Staal, 1991). In general, RNA splicing, processing of polyprotein, and fusion proteins play important roles in the retrovirus life cycle. Considering the evolutionary relationship between hepadnavirus and retroviruses, similar RNA and protein processing probably also occur in the HBV life cycle. Indeed, several pregenomic RNA-derived

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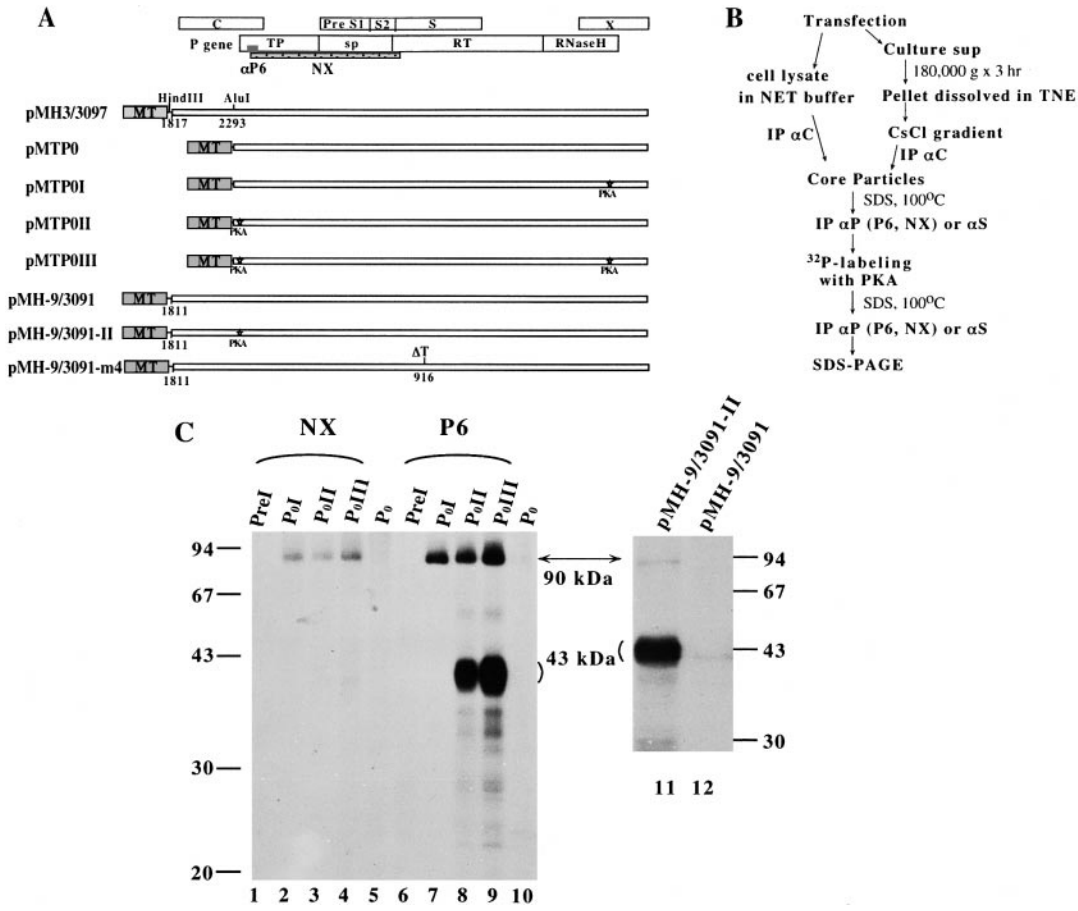

FIG. 1. Detection of a 43-kDa protein associated with HBV viral particles. (A) Schematic representation of the PKA phosphorylation site on the *P* gene of HBV. Four ORFs of HBV are shown at the top. The gray bar and stippled bar on the P ORF indicate amino acid sequences of the P protein used for generating P6 and NX antisera, respectively. Plasmid pMTP0 was constructed from pMH3/3097 (see text). Positions of the introduced PKA phosphorylation sequence in P-expressing plasmids pMTP0I, pMTP0II, and pMTP0III are indicated by *. Plasmid pMH-9/3091-m4 was derived from pMH-9/3091 by deleting a single T (nt 916). (B) A summary of methods for detection of P and its related proteins (see text for detail). (C) Analysis of the 43-kDa protein from various *pol*-expressing plasmids by immunoprecipitation assay. As represented in (B), pMTP0I (lanes 2, 7), pMTP0II (lanes 3, 8), pMTP0III (lanes 4, 9), pMTP0 (lanes 5, 10), pMH-9/3091-II (lane 11), and pMH-9/3091 (lane 12) transfected HuH-7 cell lysates were first precipitated by antiserum against the HBV core protein. Resulting immunoprecipitates were denatured by boiling, immunoprecipitated by preimmune serum (lanes 1 and 6), NX (lanes 2 to 5), or P6 (lanes 7 to 12) antisera, and labeled with [γ-^{32}P]ATP and PKA. Labeled ^{32}P proteins were denatured again, reprecipitated by the same sera, and finally subjected to 10% SDS–PAGE analysis. The positions of the 90-kDa full-length P protein and the 43-kDa protein are indicated.

spliced transcripts of HBV have been found in liver tissues or in hepatoma cell lines transfected with cloned HBV DNA (Chen *et al.,* 1989; Günther *et al.,* 1997; Su *et al.,* 1989; Suzuki *et al.,* 1989, 1990; and Terré *et al.,* 1991). Furthermore, these spliced RNAs contain ORFs encoding various P- and S-related proteins. Recently, spliced L RNA was described in duck hepatitis B virus (DHBV) and shown to be functional in early steps of the viral life cycle (Obert *et al.,* 1996). More recently, a 10-kDa protein encoded by a singly spliced RNA was reported to be expressed in HBV-infected livers and was demonstrated to be involved in the apoptosis process in transfected HuH-7 cells (Soussan *et al.,* 2000). In this study, we report a 43-kDa polymerase and surface fusion protein which was encoded by a novel HBV spliced RNA. Like the LS protein, this 43-kDa protein is a HBV structural protein incorporated into viral particles and may play a role in the viral life cycle.

RESULTS AND DISCUSSION

Construction and detection of HBV P protein containing the protein kinase A (PKA) target site

In order to detect the minute amount of the P or its related proteins of HBV, a highly sensitive method used previously for the successful detection of interferon (Li *et al.,* 1989) and HBV polymerase (Bartenschlager *et al.,* 1992) was employed. In this method, a phosphorylation site of protein kinase A was introduced into HBV *pol* gene. Subsequently, P protein or its related products from these constructs was labeled with ^{32}P by PKA phosphorylation *in vitro.* As shown in Fig. 1A, the construction

of human metallothionein promoter-controlled P-expression plasmids that carry the PKA targeting sequence at the C-terminus (pMTP0I), N-terminus (pMTP0II), and both the N- and C-termini (pMTP0III) was similar to that described before (Bartenschlager *et al.,* 1992). To produce virions carrying HBV polymerases with the PKA site, the P-expressing plasmids (pMTP0I, pMTP0II, or pMTP0III) were used to cotransfect HuH-7 cells with pMH-9/3091-m4 which carries all of the functional HBV gene products with the exception of a null mutation at the reverse transcriptase (RT) domain of polymerase (Chiang *et al.,* 1990). HBV endogenous polymerase activity assay revealed that the polymerase activity produced by pMTP0II was not affected, in which pMTP0I was reduced to one-quarter while pMTP0III was reduced to one-tenth compared to wild-type pMTP0 (data not shown).

To analyze particle-associated P or its related proteins (see flow chart of Fig. 1B), lysates or concentrated cultural media from transfected HuH-7 cells were incubated with protein A Sepharose coated with anti-core antiserum. After several washings, the immunoprecipitates were boiled in sample buffer followed by dilution with 20 vol of RIPA buffer. After centrifugation, the supernatant was immunoprecipitated again with anti-polymerase antiserum. After washings with RIPA buffer and PKA buffer, the immunoprecipitates were phosphorylated with PKA using [γ-^{32}P]ATP as a substrate (Bartenschlager *et al.,* 1992). The phosphorylated immunoprecipitates were boiled and reimmunoprecipitated with specific anti-polymerase antiserum. Finally, the immunoprecipitates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

Detection of a 43-kDa protein from secreted HBV particles

As shown in Fig. 1C, in addition to the full-length 90-kDa P protein, a considerably larger amount of doublet protein with apparent molecular weight of 43 kDa was precipitated by a P6 antiserum (Bartenschlager *et al.,* 1992), a polyclonal antibody that recognizes the N-terminal 34–51 residues of the P protein, in pMTP0II- and pMTP0III-transfected HuH-7 cell lysates (lanes 8 and 9), but not in pMTP0- and pMTP0I-transfected cell lysates (lanes 7 and 10). The 43-kDa band was specifically labeled at the introduced N-terminal PKA target site of P protein (compares lanes 7 and 10 with lanes 8 and 9). In addition, this 43-kDa doublet was not recognized by P8 antiserum against the C-terminal 813–832 residues of the P protein (data not shown, Bartenschlager *et al.,* 1992). Thus, our results indicate that the doublet-protein specifically contains the N-terminal, but not the C-terminal, portion of the P protein. A similar 43-kDa protein was reported previously by Bartenschlager and Schaller (1992) as a by-product from a transcomplementation experiment with plasmid expressing the *P* gene. Surprisingly, this 43-kDa protein could not be precipitated by a rabbit serum (NX) against the N-terminal residues 40–372 of the P protein (Lin *et al.,* 1993) that partially overlaps with the C-terminus of the P6 peptide sequence (Fig. 1C, lanes 3 and 4). This suggests that the 43-kDa protein contains the extreme N-terminal sequence of the P protein but excludes most of the TP and spacer region of the P protein. As indicated in Fig. 1B, because the transfected cell lysate was immunoprecipitated by anti-core antiserum first and then denatured and reprecipitated with antiserum against P6, this result also suggests that this 43-kDa protein, like the full-length 90-kDa P protein, should be associated with HBV core particles or core proteins in cell lysate.

To study whether the HBV genome could produce the 43-kDa protein without transcomplementation with the P-expressing plasmid containing the *P* gene, a PKA site was engineered into pMH-9/3091 (Fig. 1A, Junker *et al.,* 1987), a plasmid which is colinear with the HBV pregenomic RNA generating the plasmid pMH-9/3091-II. As shown in Fig. 1C, the production of the 43-kDa protein was again found in the pMH-9/3091-II-transfected cell (lane 11). On the contrary, the 43-kDa protein was not detected in pMH-9/3091-transfected cells (lane 12), indicating that the protein was specifically labeled at the introduced PKA target site. In this experiment, the cell lysates were directly precipitated with anti-P6 antiserum. A similar result was obtained if the cell lysates were precipitated with anti-core antiserum first and then reprecipitated with anti-P6 antiserum (as in the experiments conducted in lanes 1–10 of Fig. 1C). These results strongly suggest that the 43-kDa protein should be naturally produced in HBV-infected cells but not an artifact of the P-expressing plasmid.

To determine whether the 43-kDa protein was packaged into the secreted HBV particles, concentrated culture media from pMTP0II plus pMH-9/3091-m4-cotransfected cells were subjected to cesium chloride (CsCl) gradient centrifugation (as indicated in Fig. 1B, Huang *et al.,* 1996). Each fraction was monitored to detect the amounts of HBsAg and HBc/eAg by enzyme-linked immunosorbent assay (ELISA, Huang *et al.,* 1996) and HBV viral DNA by endogenous polymerase activity assay (Fig. 2A). For detection of the total amount of the 43-kDa protein, each fraction was boiled directly, immunoprecipitated with anti-P6 antiserum, and phosphorylated with PKA and [γ-^{32}P]ATP. The distribution of the 43-kDa proteins was found to be similar to the HBsAg profile and copeaked with the 22-nm particles (Fig. 2A, fraction No. 14). However, when anti-P6 antiserum was used to directly precipitate the intact particles which were not treated with detergent (Fig. 2C), similar amounts of the 43-kDa protein were detected from Dane particle-containing fractions (pooled fractions of 11 and 12; lane 4) and 22-nm HBsAg particles (fraction 14 on lane 6). En-

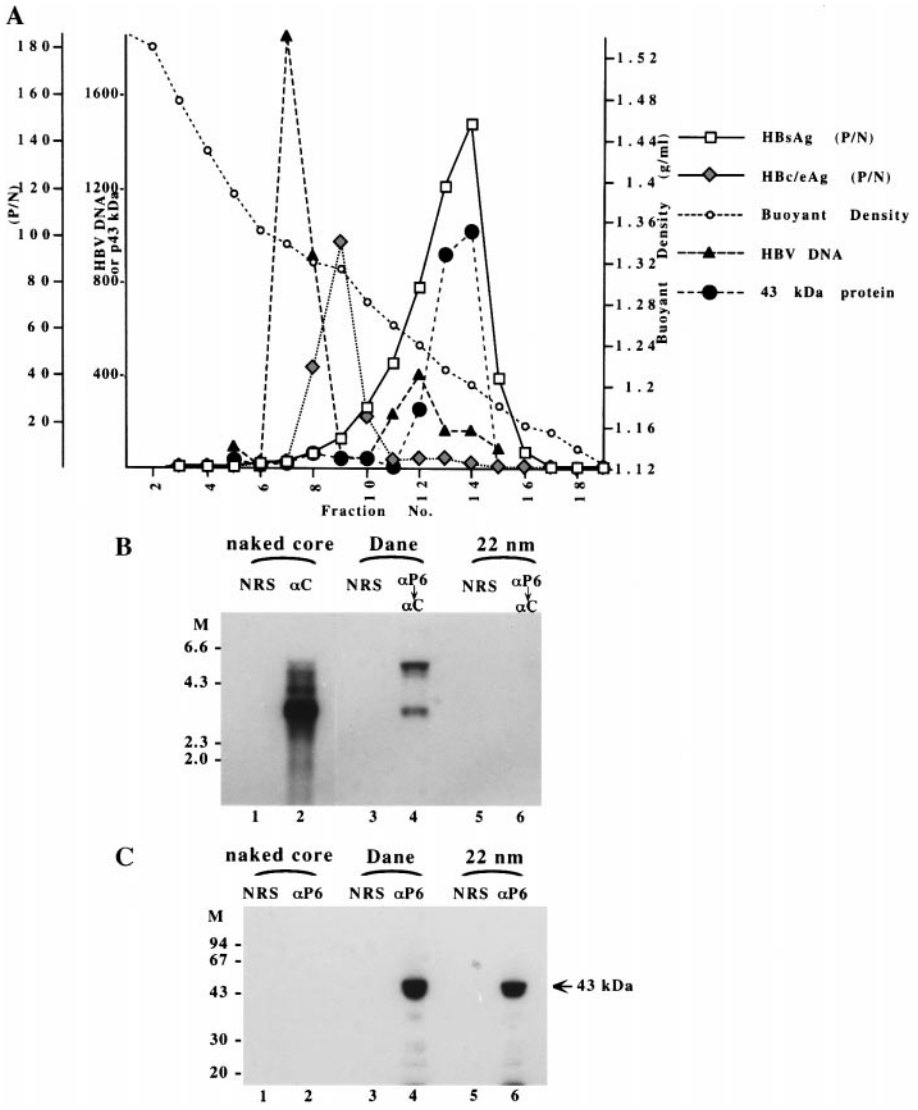

FIG. 2. Association of 43-kDa proteins with secreted HBV viral particles. Secreted viral particles from pMTP0II- plus pMH-9/3091-m4-transfected cells were separated by CsCl gradient centrifugation. (A) Profiles of HBsAg, HBc/eAg, HBV DNA, and 43 kDa in a CsCl gradient. HBsAg (open square) and HBc/eAg (gray diamond) were measured by ELISA. The value of P/N represents the sample absorbancy value divided by the negative control absorbancy value. Buoyant density (open circle, g/ml) was converted from the refractive index. The amounts of HBV viral DNA (solid triangle) were detected by HBV endogenous polymerase activity assay using anti-core immunoprecipitates from the NP-40-treated fraction and then were quantitated by a densitometer. The total amount of the 43-kDa protein (solid circle) in each fraction was boiled and detected by immunoprecipitation and ^{32}P-phosphorylation and then was quantitated by a densitometer. (B and C) Association analyses of the 43-kDa protein in various viral particles. (B) HBV viral DNA and (C) the 43-kDa protein were detected in three kinds of HBV viral particles from CsCl fractions. Naked core particles in buffer-diluted fractions 7 and 8 were immunoprecipitated with normal serum (NRS, lane 1) or anti-core (αC, lane 2) antiserum. Dane particles or 22-nm particles in buffer-diluted fractions 11 and 12 or fraction 14 were immunoprecipitated with normal serum (NRS, lanes 3 and 5) or anti-P6 antiserum (αP6, lanes 4 and 6). After being washed, samples were divided into two parts (B and C). For detection of viral DNA in (B), anti-P6-immunoprecipitates were treated with NP-40 and diluted, and the supernatants were reimmunoprecipitated with anti-core antiserum and HBV endogenous polymerase activity assay was performed as described under Materials and Methods. The exposure time of lanes 3 to 6 is eightfold longer than in lanes 1 and 2 in Fig. 2B. For detection of the 43-kDa proteins in (C), anti-P6 immunoprecipitates were boiled, reprecipitated, and ^{32}P-phosphorylated as described under Materials and Methods and in Fig. 1.

dogenous polymerase activity assay revealed that HBV DNA was detected in fractions of naked cores and Dane particles but not in fractions of 22-nm particles (Fig. 2B). These results suggest that: (i) the 43-kDa protein is present in Dane particles and 22-nm HBsAg particles; (ii) a P6 epitope of the 43-kDa protein may be exposed on the surface of these particles; (iii) similar to the LS protein, the 43-kDa protein was enriched in Dane particles.

To determine the strength of the association between the 43-kDa protein and HBV core particles, the secreted HBV particles were first treated with 1% NP-40 to disrupt 22-nm HBsAg particles and remove envelopes from the Dane particles. The core particles were collected by

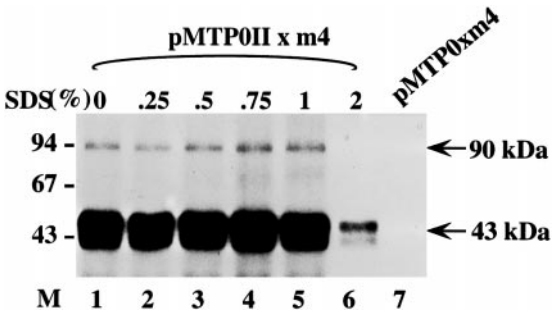

FIG. 3. Characterization of the association between the 43-kDa and HBV core proteins. The culture supernatant collected from pMTP0II-plus pMH-9/3091-m4-transfected HuH-7 cells was treated with 1% NP-40. The core particles were then collected by ultracentrifugation through a 20% sucrose cusion and were dissolved in TNE buffer. After treatment with various concentrations of SDS at room temperature for 30 min, samples were diluted with TNE buffer (final to 0.1% SDS) and were immunoprecipitated with anti-core antiserum. After reprecipitation with P6 antiserum and labeling with PKA and [γ-^{32}P]ATP as described in the legend to Fig. 1C (lanes 1–10), immunoprecipitated samples were analyzed by 10% SDS–PAGE. The positions of the 90-kDa full-length P protein and the 43-kDa protein are indicated.

ultracentrifugation through a 20% sucrose cusion and treated with TNE buffer containing 0.25 to 2% SDS, followed by immunoprecipitation with anti-core antiserum. After reprecipitation with P6-antiserum and labeling with PKA and [γ-^{32}P]ATP as described, our results indicate that the association between the 43-kDa and HBV core proteins was very strong based on the finding that the association was stable even up to 1% SDS (Fig. 3). The quantity of the 43-kDa protein and 90-kDa polymerase was compared using a densitometer. We found that the amount of the 43-kDa protein was about 10-fold higher than that of the 90-kDa polymerase (Fig. 3, lane 1). Since each virion was reported to contain one molecule of polymerase (Bartenschlager and Schaller, 1992), the estimated number of the 43-kDa protein molecules on each virion should be approximately 10. However, the actual number of the 43-kDa protein molecules associated with each virion may be higher, since the 43-kDa protein may be present on the viral envelope in addition to that associated with core particles.

The 43-kDa protein contains both P and preS-S antigen epitopes

The fact that the 43-kDa protein can only be recognized by P6 antiserum (reacted with amino acid residues 34 to 51 of P, Fig. 1A) but not by NX antiserum (reacted with amino acid residues 40 to 372 of P) or by P8 antiserum (reacted with the last C-terminus sequence of P) implied that the C-terminus of the 43-kDa protein was likely encoded by other ORFs extrinsic of the P gene. To test the possibility that the 43-kDa protein contains the epitope of the surface protein, sequential immunoprecipitation was performed on cell lysates from pMTP0- or pMTP0II-transfected HuH-7 cells which were immunoprecipitated with goat anti-HBsAg antiserum or with P6 antiserum (Fig. 4A) and then reprecipitated with P6 or goat anti-HBsAg antiserum. As shown in Fig. 4A, the goat anti-HBsAg antiserum-precipitated 43-kDa protein could be reprecipitated by P6 antiserum (lanes 2 and 4). In the converse experiment, the 43-kDa protein first precipitated by P6 antiserum could also be reprecipitated with DAKO antiserum (lanes 10 and 12). The control experiments showed that the 43-kDa protein could not be detected from pMTP0-transfected HuH-7 lysates (lanes 5 to 8 and 13 to 16) nor precipitated by normal sera from pMTP0II-transfected HuH-7 lysates (lanes 1, 3, 9, and 11). The amount of the 43-kDa protein sequentially precipitated by DAKO antiserum (lane 2) was much weaker than that precipitated by DAKO followed by P6 antiserum (lane

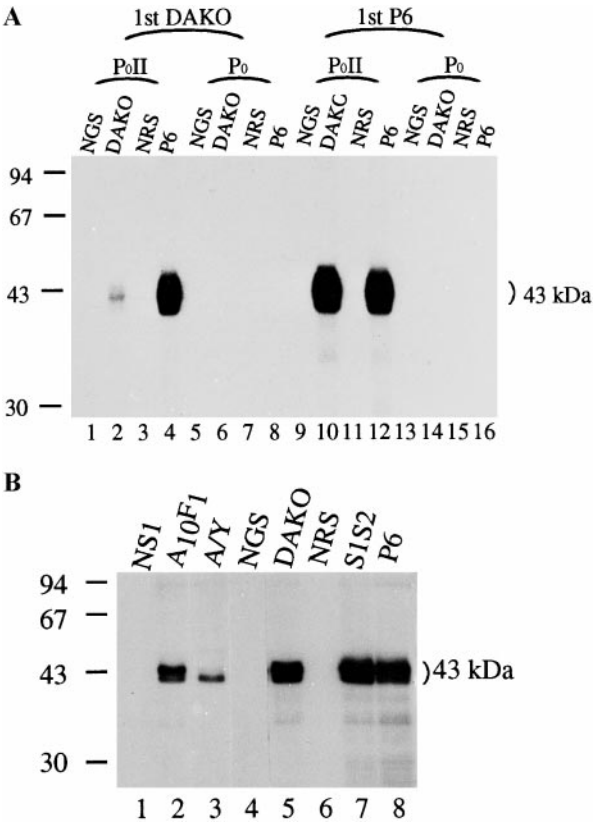

FIG. 4. The 43-kDa protein contains both antigenic determinants of the HBV P and S protein. (A) Characterization of the 43-kDa protein by immunobiochemical assay. Lysates from pMTP0- or pMTP0II-transfected HuH-7 cells were first immunoprecipitated with DAKO (lanes 1 to 8) or P6 (lanes 9 to 16) antisera. Analysis procedures were performed as for Fig. 1B using antisera as indicated at the top of the lanes. The loading amounts in lanes 10 and 12 were one-half and one-eighth. (B) Immunoreactivity of the 43-kDa protein with various HBV surface antisera. The 43-kDa protein was first precipitated by anti-P6 antiserum. After being labeled with ^{32}P and boiled, the sample was aliquoted and reprecipitated with $A_{10}F1$ (lane 2), A/Y (lane 3), DAKO (lane 5), S1S2 (lane 7), P6 (lane 8), and control sera (lanes 1, 4, and 6). The exposure time of lanes 1 to 3 is 10-fold longer than that of lanes 4 to 8. All of the immunoprecipitates were subjected to 10% SDS–PAGE and autoradiography.

4). This discrepancy may arise from competition of the 43-kDa protein with a large amount of dissociated HBsAg. To sum up, the results suggest that the 43-kDa protein contains epitopes of both P and S proteins.

To further understand whether the 43-kDa protein contains domains of LS, MS, and SS protein, a panel of HBV surface protein antisera was used to map the epitopes of the S protein portion of the 43-kDa protein (Fig. 4B). These antisera include the goat anti-HBsAg antiserum (DAKO) that recognizes the whole denatured HBV surface proteins including SS, MS, and LS protein; S1S2, a rabbit antiserum that was raised against preS1–preS2 domains of the surface protein (Sheu and Lo, 1995); $A_{10}F_1$ monoclonal antibody that recognizes the SS domain; and A/Y antiserum (Lee *et al.,* 1984) that was shown to react with the unglycosylated but not glycosylated form of SS (data not shown). When the immunoprecipitates of the pMTP0II-transfected cell lysates obtained with P6 antiserum were reprecipitated with these anti-HBsAg antisera, the 43-kDa protein could be reprecipitated by DAKO (lane 5), S1S2 (lane 7), $A_{10}F_1$ antisera (lane 2), and A/Y antiserum (lane 3) (Fig. 4B). These results indicate that the 43-kDa protein contains the epitopes of the preS1–preS2 and SS region of the surface antigen. Interestingly, A/Y antiserum precipitated exclusively the lower band of the 43-kDa doublet. This result explains the possibility that the higher band of the 43-kDa doublet might be a glycoprotein, a modification that was present on surface proteins (Nassal, 1996).

The 43-kDa protein is a glycoprotein

To determine whether the 43-kDa protein is glycosylated, the P6 antiserum-precipitated and ^{32}P-labeled 43-kDa protein was treated with endoglycosidase H (EndoH) or peptide:N-glycosidase F (PNGaseF) to remove the N-linked oligosaccharide from the glycoprotein. As shown in Fig. 5A, the apparent molecular weight of the 43-kDa protein (lanes 3 and 7) on SDS–PAGE was shifted to 41 kDa after EndoH (lane 1) or PNGaseF (lane 5) treatment. A similar result was obtained from cells treated with tunicamycin, a glycosylation inhibitor, as demonstrated in Fig. 5B (lanes 2 and 4). Taken all together, these results indicate that the 43-kDa protein is glycosylated.

The 43-kDa protein contains the C-terminal half of the *SS* gene

To further confirm that the 43-kDa protein contains the *S* ORF, the C-terminal half of the *SS* gene of pMTP0II was deleted and replaced by a *Xba*I linker which contained a stop codon at three different frames to generate pMTP0IIΔCS (Fig. 5C). As shown in Fig. 5C, the band corresponding to the truncated *P* gene product (PΔCS) was now shifted from 90 to 50 kDa and the 43-kDa doublet protein was shifted to approximately 33–31 kDa (lane 3; the 31-kDa protein in lane 4 was the unglycosylated form of P-SΔCS). We obtained the similar conclusion from another pre-S1 deletion mutant pMTP0IIΔBE (nt 2904–1, Radziwell *et al.,* 1990). In conclusion, these results provide strong evidence that the 43-kDa protein contains the pre-S to the carboxyl terminal half region of the *SS* gene.

The 43-kDa protein is encoded by a HBV spliced RNA characterized by joining the *P* ORF to the *S* ORF

Immunochemical analyses of the 43-kDa protein revealed that it contains both the antigenic determinants of the polymerase and the surface protein. A possible mechanism to account for the origin of the 43-kDa protein is that it may be encoded by a spliced RNA characterized by the adjoining *P* and *S* genes. Therefore, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was conducted to test for the possibility of the existence of a spliced transcript. Immunochemical analyses suggest that the 43-kDa protein should be initiated from the initiation codon of P and immediately fused to the surface protein ORF. Thus, two primers were designed to amplify the DNA fragment that covered the putative spliced junction as depicted in Fig. 6A. The sense primer P1 was close to the initiation site of the P protein and the antisense primer P2 was located at the *SS* region. The previously reported single or double spliced RNA (Chen *et al.,* 1989; Günther *et al.,* 1997; Su *et al.,* 1989; Suzuki *et al.,* 1989, 1990; and Terré *et al.,* 1991) would not be amplified by these primers because the primers P1 and P2 were within the intron region of these spliced RNAs. The RNA isolated from pSHH2.1- (Chang *et al.,* 1987) and pMH-9/3091-transfected cells was subjected to RT-PCR using these two primers. In addition to the 1.0-kb product corresponding to the unspliced-pregenomic RNA, a new 0.6-kb PCR product was clearly amplified by these primers in samples from pSHH2.1-, pMH-9/3091-, and pMTP0-transfected cells (data not shown). Both the 1.0- and the 0.6-kb bands were not detected in samples from untransfected cells. These 0.6-kb products were cloned into the pT-Adv vector and sequenced. It was found that the sequence of HBV at nt 2902 was now joined to nt 2447, skipping 454 nts, as demonstrated in Fig. 6B. The same deletion was found in cDNA from a hepatocellular carcinoma cell line, PLC/PRF/5 (Ziemer *et al.,* 1985). Deduced amino acids sequence of the 0.6-kb product revealed that the 47th amino acid of the P protein was fused to the 19th amino acid of the LS protein. Sequence alignment showed the presence of consensus splice donor and acceptor sites at junctions (Fig. 6B). Furthermore, these splicing donor and acceptor sites were conserved among various subtypes of HBV (Fig. 6C). To examine whether the newly identified HBV spliced RNA contained spliced regions other than 2447/2902, primers near the 5′ or 3′ ends of

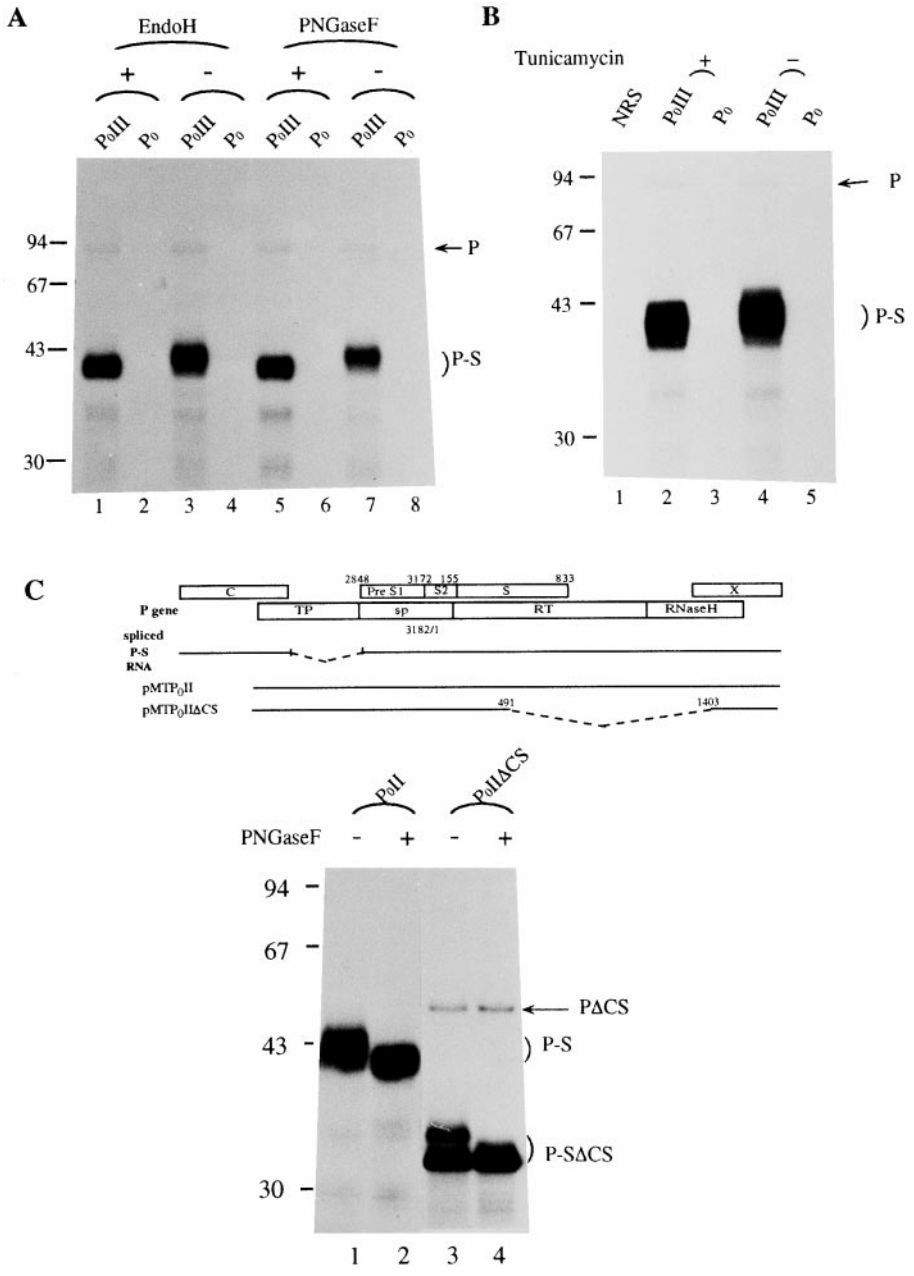

FIG. 5. The analysis of glycosylation of the 43-kDa protein. (A) Glycosylation analysis of the 43-kDa protein *in vitro*. Following precipitation with anti-P6 antiserum and ^{32}P-labeling, samples were denatured, equally divided, and incubated with endoH (lanes 1 and 2) or with PNGaseF (lanes 5 and 6) or with no enzymes (−, lanes 3, 4, 7, and 8). (B) The analysis of glycosylation of the 43-kDa protein *in vivo*. pMTP0- or pMTP0III-transfected HuH-7 cells at 2 days posttransfection were treated with 20 μg/ml tunicamycin (lanes 2 and 3) or equal volumes of 50% EtOH (as negative control; lanes 4 and 5). The 43-kDa protein was obtained and analyzed essentially as for Fig. 1C (lanes 11 and 12). (C) Deletion analysis of the 43-kDa protein. (Top) Schematic representation of the deletion regions of construct pMTP0IIΔCS. Anti-P6 immunoprecipitates from pMTP0II- (lanes 1 and 2) or pMTP0IIΔCS- (lanes 3 and 4) transfected HuH-7 cell lysates were treated with PNGaseF (lanes 2 and 4) or with no enzymes (−, lanes 1 and 3). The truncated P proteins or P–S proteins are indicated by PΔCS or P-SΔCS. All of the immunoprecipitates from (A) to (C) were finally subjected to 10% SDS–PAGE.

the HBV pregenomic RNA and primers containing the 2447/2902 splice junction were used. For the mapping of the 5′-end, we used a reverse primer harboring the junction sequences of 2447/2902 paired with a primer of the very 5′-end of pregenomic RNA. The sequencing analysis revealed that a single band was amplified and there was no spliced region (data not shown). To explore the 3′-end of this splice RNA, we used a splicing junction 2447/2902 containing primer and reverse primer near the 3′-end of the HBV genome. Two PCR products were found. One product contained no other deletion and the other had a deletion at nts 2985 to 489. These results indicate that the transfected HuH-7 cells produce two spliced RNAs with one containing a single spliced region

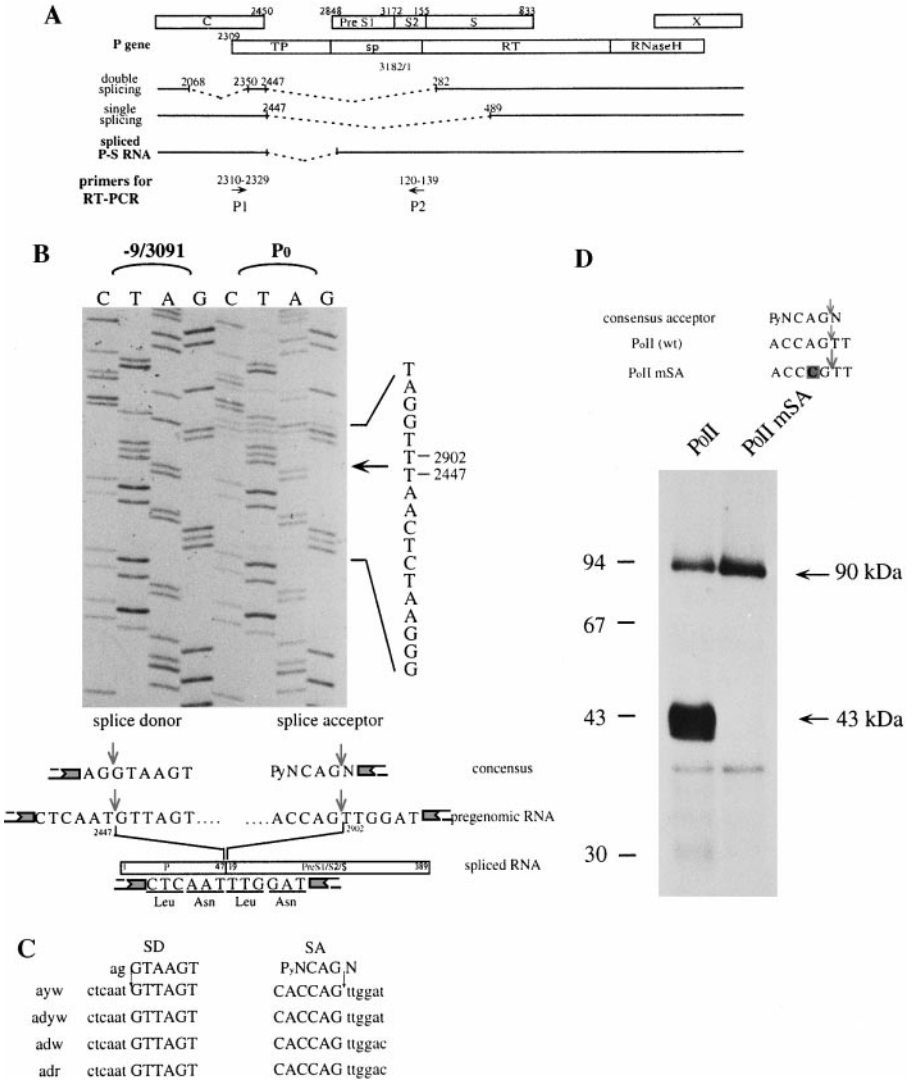

FIG. 6. Cloning and characterization of a novel spliced cDNA. (A) Diagram of the putative spliced RNA and the positions of the primers used in this study. HBV gene organization and the nucleotide sequences corresponding to their 5′ and 3′ ends of individual genes are indicated at the top. The published single (Su *et al.,* 1989) and double spliced RNAs (Chen *et al.,* 1989) are illustrated in the middle. The spliced P–S RNA represents the putative splice RNAs. Arrows stand for primers (P1 and P2) and the nucleotide number of both the 5′-end and the 3′-end of primers as well as the junction of single and double splicing RNAs are also indicated. The dashed line represents the introns. (B) Sequence determination and alignment analysis of the spliced cDNA. The cDNA was amplified by RT-PCR from total RNAs of pMH-9/3091- or pMTP0-transfected cells. PCR fragments were cloned into the pT-Adv vector. Clones that contain putative spliced cDNA fragments were sequenced. The positions (arrow) and sequences of the splice junction are indicated. (Bottom) RNA alignment analysis of the splice consensus sequence (top) and pregenomic sequence of HBV (middle). Vertical arrows indicate the splice donor and acceptor sites. The black bars with arrows plus the sequences up or down to the vertical arrows represent exons. The spliced junction and its deduced amino acids are indicated at the bottom. (C) Sequence alignment at the splice donor and acceptor site sequences between four subtypes of HBV. (D) The production of the 43-kDa protein by mutation analysis. (Top) Conserved AG dinucleotides of the splice acceptor site in the wild-type pMTP0II were mutated to CG, resulting in the generation of the plasmid of pMTP0IImSA (the mutated nucleotide is underlined). The analysis of the 43-kDa protein was performed as in Fig. 1C.

of 2447 to 2902 and the other containing a double spliced region of 2985 to 489, in addition to the 2447 to 2902. Since the latter RNA cannot be amplified by our reverse primer P2 (nts 120 to 139) and the 43-kDa protein has epitopes of pre-S and SS domains of the surface protein (Figs. 4B and 5C), the 43-kDa protein could not possibly be encoded by such doubly spliced RNA. To sum up, these results suggest that a HBV RNA, with a single splice at nts 2447 to 2902, should be the mRNA encoding for the 43-kDa protein.

To substantiate the finding that the 43-kDa protein is encoded by a spliced RNA as described above, the conserved nucleotide at nt 2902 in the splice acceptor site of pMTP0II was changed from A to C to generate the construct pMTP0IImSA. This mutation should result in a silent mutation on the P protein. As shown in Fig. 6D, the

full-length P protein was clearly expressed in the pMTP0II- (lane P0II) and pMTP0IImSA- (lane P0IImSA) transfected cells. However, the production of the 43-kDa protein was abrogated in pMTP0IImSA compared to the wild type (pMTP0II). A similar result was obtained when the conserved splice donor site at nt 2446 was mutated from A to C (data not shown). Furthermore, using [^{35}S]methionine (Met) *in vivo* labeling and immunoprecipitation, we detected a 43-kDa doublet protein from pMTP0sp- (the spliced cDNA was cloned into plasmid pMTP0) transfected cells. Like the ^{32}P-labeled P–S protein, this [^{35}S]Met doublet protein was also precipitated with anti-P6 antiserum and reprecipitated by anti-surface antiserum (data not shown). Therefore, we conclude that the 43-kDa protein is encoded by this single spliced RNA.

The 43-kDa P–S fusion protein is able to substitute for the LS protein in virion maturation

The P–S fusion protein containing the majority of the LS protein and N-terminal truncation up to residue 91 (subtype ayw) of the LS protein still allowed virion secretion (Bruss *et al.,* 1994); therefore, we examined whether the P–S fusion protein was able to substitute for the function of the LS protein in virion maturation. To generate the LS amber mutant plasmid (pMH-9/3091LS$^-$), the ATG start codon of LS in pMH-9/3091 was converted to ACG. This mutation did not alter the amino acid sequence of polymerase (Ueda *et al.,* 1991). A P–S fusion protein expressing plasmid, pMTP0sp, was constructed by replacing the fragment of pMTP0 with 2447/2902 spliced cDNA. To examine virion production, collected particles from the culture media of cells transfected with pMH-9/3091 or pMH-9/3091LS$^-$ or cells cotransfected with pMH-9/3091LS$^-$ and pMTP0sp (4:1) were subjected to CsCl gradient fractionation and the HBV viral DNA in each fraction was monitored by HBV endogenous polymerase activity assay. As reported previously (Gerelsaikhan *et al.,* 1996), viral particles from the wild-type genome could be separated into naked core particles (labeled with naked) and Dane particles (labeled with Dane) as shown in Fig. 7A. Loss of LS protein expression failed to produce virions (Bruss *et al.,* 1994) (Fig. 7B, fractions 9–11, a density of about 1.26 to 1.238 g/ml), but these transfected cells still could produce naked nucleocapsids (fractions 3 to 6, a density of about 1.36 to 1.314 g/ml). Upon cotransfection with P–S fusion protein expressing plasmid pMTP0sp, particles containing HBV DNA with a density of about 1.248 to 1.234 were rescued (Fig. 7C, fractions 9–11). Reproducible results were also obtained using immunoprecipitation methods to purify Dane particles by antiserum against HBsAg (data not shown; methods as described in Gerelsaikhan *et al.,* 1996; and Huang *et al.,* 1996). In summing up the above, these data indicate that the 43-kDa P–S fusion proteins are present in Dane particles and are able to substitute for the LS protein in virion maturation.

Although there are many HBV spliced RNAs which have been identified (Chen *et al.,* 1989; Günther *et al.,* 1997; Su *et al.,* 1989; Suzuki *et al.,* 1989, 1990; and Terré *et al.,* 1991); only one of their protein products has been reported, i.e., the 10-kDa protein (Soussan *et al.,* 2000). This 10-kDa protein was translated from the first characterized singly spliced RNA (Su *et al.,* 1989). Apparently, it was reported not to be involved in the regulation of viral transcription and replication; however, this protein could induce apoptosis in transfected cells. Our results show that another protein, a polymerase/surface fusion glycoprotein, is encoded by a spliced RNA. The glycoprotein was demonstrated to be a structural protein of Dane particles and 22-nm subviral particles. Each virion was estimated to contain about 10 molecules of the 43-kDa protein. Since 40 to 80 LS molecules were reported to be present in each virion (Heermann *et al.,* 1984), the number of the 43-kDa protein molecules per HBV virion is approximately one-fourth to one-eighth that of the LS protein. It is interesting to note that the P–S protein was detected in membrane-containing viral particles, 42-nm Dane particles, and 22-nm subviral particles, but not in naked core particles or culture medium (Fig. 2). However, the P–S protein does interact with core protein inside cells (Fig. 1). We do not know the mechanism by which naked core particles are transported outside of the cells. Probably, core particles that are destined to depart from the cells (as naked core particles) are sequestered in an environment where interaction with the P–S protein is not permissible. On the contrary, membrane-containing particles can readily interact with surface proteins, including the P–S protein, on the exterior of the endoplasmic reticulum before assembling into mature viruses. The interaction could not be dissociated when cells were lysed with NP-40 or during CsCl gradient centrifugation. This explanation may account for the discrepancy that the P–S protein can interact with the core protein within the cell, but cannot associate with naked core particles.

Similar to our 43-kDa protein, an L protein of DHBV was recently reported by Obert *et al.* (1996). This L protein of DHBV is also encoded by a spliced RNA derived from the pregenomic RNA of DHBV. The L protein is identical to the DHBV envelope protein but with additional function in controlling a distinct role in an early stage of the viral life cycle. Structurally, the 43-kDa protein is similar to the L protein with the exception that the N-terminus of the LS protein is replaced by the first 47 amino acids of the N-terminus of the P protein. This replacement leads to the loss of the myristylation site, a site for posttranslational modification at the extreme N-terminus of the LS protein. Similar to the 10-kDa protein of HBV and the L protein of DHBV, the 43-kDa protein had no effect on viral replication in HuH-7 cells transfected with pMH-9/3091mSA mutant plasmid (data not shown).

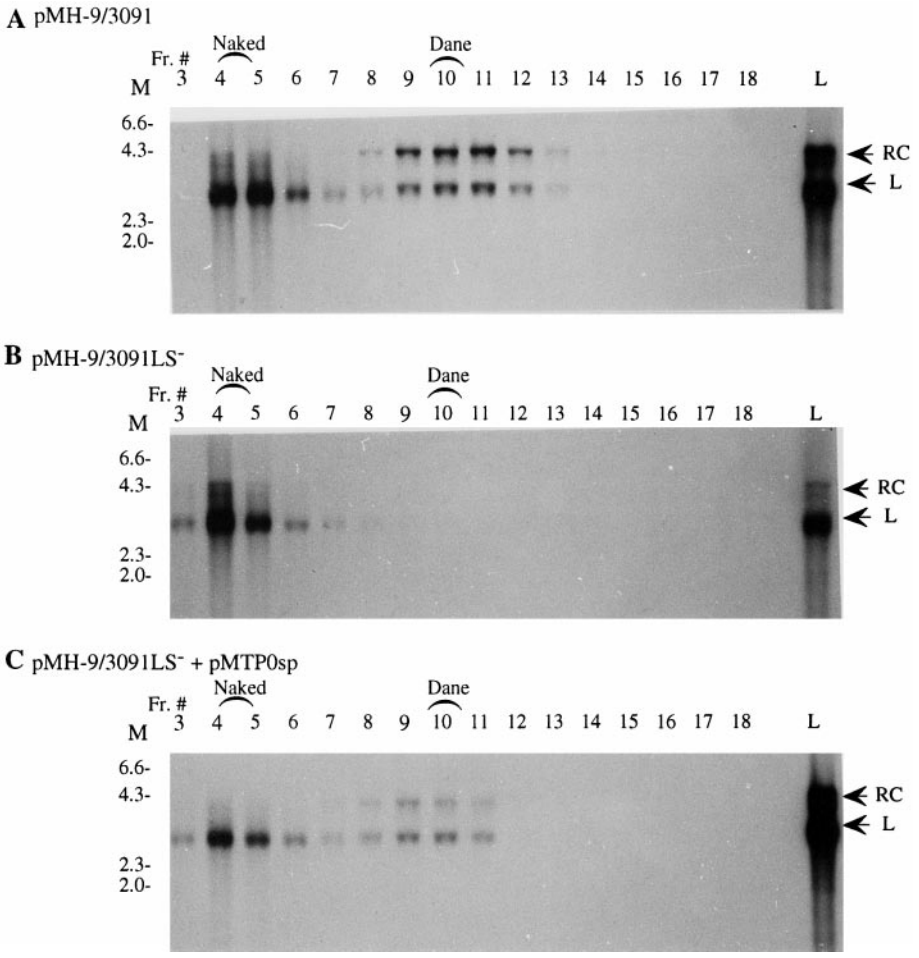

FIG. 7. The 43-kDa P–S protein is able to substitute for the LS protein in virion formation. After CsCl density gradient fractionation of cultural media from pMH-9/3091- (top), pMH-9/3091LS$^-$- (middle), or pMH-9/3091LS$^-$ plus pMTPosp- (4:1) (bottom) transfected cells, DNA contents in naked viral particles and virions were detected by endogenous polymerase activity assay as in Fig. 2A. Fraction numbers (Fr #) are shown at the top of each lane. The relaxed circular form (RC) and linear form (L) are indicated on the right side, and the fractions contain naked core particles (naked) or Dane particles (Dane) are indicated at the top.

Unlike the DHBV, an animal or *in vitro* infection model for HBV is not readily available to test whether the 43-kDa protein plays a role in the HBV life cycle. Therefore, the possibility that the 43-kDa proteins may be merely present in HBV Dane and 22-nm subviral particles without biological functions cannot be completely ruled out.

Despite the difficulty, our studies clearly demonstrated that the 43-kDa protein could substitute for the LS protein for HBV maturation (Fig. 7). More importantly, many characteristics were found to be similar between both the LS protein and the P–S protein. First, since our 43-kDa protein was able to interact with the core proteins in the cell lysate (Fig. 1 and data not shown), it resembles the interaction between the LS and the core protein as demonstrated previously (Dyson and Murray, 1995; Poisson *et al.,* 1997). Furthermore, the interacting domains of both the LS and the 43-kDa protein were mapped to the pre-S1 region (nts 2904 to 1 for the P–S protein) (data not shown; Dyson and Murray, 1995). These results may account for why the 43-kDa protein is able to substitute for the LS protein in virion formation. Second, like the LS protein, the 43-kDa P–S protein was relatively more enriched in Dane particles than in 22-nm HBsAg particles (Fig. 1 and Nassal, 1996). Finally, previous results have demonstrated the possibility that the LS proteins of HBV interact with their receptors on hepatocytes (Pontisso *et al.,* 1989). The high degree of homology between the 43-kDa protein and the LS protein of HBV suggests that the 43-kDa protein may also be involved in the entry of HBV to hepatocytes.

MATERIALS AND METHODS

Cell culture, transfection, and preparation of cell lysate or secreted viral particles

HuH-7 cells were grown at 37°C in Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% nonessential amino acid, and 25 μg/ml fungizone under 5% CO_2. Forty micrograms of

plasmid DNA (20 μg of each plasmid for cotransfection) was used to transfect 1×10^7 cells/150-mm petri dish by the calcium phosphate coprecipitation method as described previously (Chang *et al.,* 1987). Three days posttransfection, cells were harvested and lysed with NET buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40]) containing 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 2 μg/ml leupeptin, and 2 μg/ml aprotinin (Boehringer Mannheim, Germany). Then the cell lysates were clarified by centrifugation (17,000*g*, 4°C, Sigma 2K15) before immunoprecipitation. Secreted viral particles from the culture fluid of transfected HuH-7 cells were precleared and concentrated by ultracentrifugation at 180,000*g* for 3 h as described previously (Huang *et al.,* 1996). Finally, the pellets were dissolved in TNE buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA).

Plasmid constructions

Standard methods were used for plasmid construction (Sambrook *et al.,* 1989). The mutated sequences were generated by site-directed mutagenesis (Promega, Madison, WI) or a standard jumping PCR procedure and then were confirmed by sequencing using the dideoxy termination method (Sanger *et al.,* 1977).

In this study, the unique *Eco*RI site on the HBV genome (ayw subtype) was numbered nucleotide 1. Plasmid pMH-9/3091 contains an overlength HBV genome, which is under the control of the human metallothionein promoter (Junker *et al.,* 1987). The polymerase defective mutant, pMH-9/3091-m4, was used for complementing core proteins in the cotransfection system (Chiang *et al.,* 1990). The polymerase expression plasmid, pMTP0, was generated by deletion of the *Hin*dIII–*Alu*I (nt 2291) fragment of pMH3/3097 by multiple steps. First, the *Alu*I fragment (nts 2291 to 2992) of pMH3/3097 was inserted into the *Hind*III site located in front of the HBV sequence on the same plasmid. The resulting plasmid was digested with *Bsp*MII (nt 2330) followed by self-ligation. The upstream flanking sequence of the initiation codon was changed to CGCATG (to create a Kozak sequence) according to the manual of the pSELECT-1 site-directed mutagenesis kit (Promega) using an oligonucleotide of 5′-GCTTATAGACCACCGCATGCCCCTATCCT-3′ (nt 2291 to 2319). To construct the polymerase containing the protein kinase A targeting sequence, the oligonucleotides containing the PKA consensus sequences were introduced into pMTP0 by site-directed mutagenesis as described above. The oligonucleotide, 5′-1512-CGACCGACCACGCGTCGCACCTCTCTT-1538-3′, was used to generate pMTP0I; pMTP0II was produced by the oligonucleotide 5′-2325-CACTTCCGGAGACTATCGATGTTAGACGACGAGG-2358-3′; pMTP0III was generated to contain both PKA targeting sites on the *P* gene using both oligonucleotides. For generation of pMH-9/3091-II, a PKA target site from pMTP0II was engineered into pMH-9/3091 by replacing the *Bsp*MII to *Mun*I (nts 2330 to 2629) fragment of pMTP0II. To generate the construct pMTP0IImSA, the conserved splice acceptor site at nt 2900 was changed from A to C by site-directed mutagenesis with the oligonucleotide 5′-2890-TTCCCGACCACCCGTTGGATCCAGC-2914-3′. All of the oligonucleotides in which the sequences mismatch to the wild-type sequence are underlined. pMTP0IIΔCS was constructed by replacement of the *Bam*HI–*Bam*HI (nts 490–1402) fragment of pMTP0II with the *Xba*I linker which contains a stop codon at three different frames (Stratagene, La Jolla, CA). A P–S fusion protein expressing plasmid, pMTP0sp, was constructed by replacing the *Bgl*II to *Eco*RI (nts 2424 to 1) fragment of pMTP0 from the 2447/2902 spliced cDNA. To generate the LS amber mutant plasmid pMH-9/3091LS$^-$, the ATG start codon of LS in pMH-9/3091 was converted to ACG with the oligonucleotide 5′-2839-ATCTACAGCACGGGGCAGAATCTT-2862-3′ by a standard jumping PCR procedure.

Antisera

Anti-P6 is a rabbit serum which was generated by the peptide (amino acid residues 34 to 51) of the polymerase (Bartenschlager *et al.,* 1992). NX antisera were generated by several injections using SDS–PAGE-purified glutathione S-transferase-fused P fragment that corresponds to the amino acid residues of 40 to 372 of the polymerase (Lin *et al.,* 1993). A10F$_1$ and A/Y surface monoclonal antibodies were kindly provided by Dr. Lee (Lee *et al.,* 1984). DAKO is a goat polyclonal antiserum against the surface protein that was purchased from DAKO Company (DAKO Corp., Carpinteria, CA). S1S2 is a rabbit polyclonal antiserum against pre-S1–pre-S2 domains of the surface protein (Sheu and Lo, 1995). Rabbit antiserum against the core protein was generated by injections of HBV core proteins expressed from *Escherichia coli.*

Immunoprecipitation and radioactive labeling of proteins with PKA

The precleared cell lysates or the concentrated culture media were reacted with protein A Sepharose coated with antibodies against polymerase or surface protein (see Fig. 1B and text). After several washings with NET buffer or RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and PKA buffer (20 mM Tris–HCl, pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$), the immunoprecipitates were phosphorylated by PKA using [γ-^{32}P]ATP (New England Nuclear, Boston, MA) as substrate (Bartenschlager *et al.,* 1992). The phosphorylated immunoprecipitates were washed with PBS and boiled with sample buffer (200 mM Tris–HCl, pH 8.0, 5 mM EDTA, 2% SDS, 1% 2-mercaptoethanol [2-ME], 10% sucrose, and 0.1% bromophenol

blue). The supernatant was diluted with RIPA buffer and reimmunoprecipitated with specific anti-polymerase antiserum. Finally, the immunoprecipitates were boiled in sample buffer and subjected to 10% SDS–PAGE and autoradiography. For analysis of the core-associated polymerase or the P–S protein, the cell lysates or concentrated culture media were first reacted with rabbit antiserum against the core protein. They were then washed, boiled, and reprecipitated with antisera against polymerase as described above.

Separation of HBV particles by cesium chloride gradients

Concentrated viral particles were mixed with 10 ml of CsCl solution (final concentration 1.24 g/ml) and centrifuged at 150,000*g* for 44 h in a Beckman SW41 rotor (Huang *et al.,* 1996). Four hundred microliters of each fraction was collected from the bottom of the tubes and the density was determined with a refractometer. Surface and HBV c/e antigens were determined by ELISA (Huang *et al.,* 1996). In Figs. 2B and 2C, pooled fractions were diluted with TNE buffer and were immunoprecipitated with anti-P6 antiserum to further purify the Dane particles and 22-nm HBsAg particles. For detection of the 43-kDa protein (Fig. 2C), the immunoprecipitates were boiled, diluted, and reprecipitated with anti-P6 antiserum and phosphorylated as described above. For detection of the HBV DNA in Dane particles in Fig. 2B, the anti-P6 immunoprecipitates were treated with TNE buffer containing 2% NP-40 to release the viral nucleocapsids (Huang *et al.,* 1996) and diluted with TNE buffer, and then the supernatants were immunoprecipitated with antiserum against the core proteins. After several washes, the samples were subjected to HBV endogenous polymerase activity assay.

HBV endogenous polymerase activity assay

In Figs. 2A and 7, CsCl fractions were directly treated with TNE buffer containing 2% NP-40 and then diluted and immunoprecipitated with rabbit antiserum against the core. After being washed, immunoprecipitates were incubated in polymerase buffer (50 mM Tris–HCl, pH 7.5, 40 mM NH_4Cl, 10 mM $MgCl_2$, 1% NP-40, 2% 2-ME, 12.5 μM each dATP, dGTP, dTTP, 80 μCi [α^{32}P]dCTP (5000 Ci/mmol), for 3 h at 37°C (Chang *et al.,* 1987; Kaplan *et al.,* 1973). The unlabeled dCTP (12.5 μM) was then added for filling the remaining gaps. After incubation and washings, proteinase K (200 μg/ml, Merck, Germany) and SDS (0.5%) were added for 2 h at 37°C. Then labeled HBV DNA were extracted and subjected to 1.2% agarose gel electrophoresis and autoradiography.

Glycosylation analyses

The transfected HuH-7 cells at 2 days posttransfection were treated with 20 μg/ml tunicamycin (in 50% ethyl alcohol (EtOH]) or 50% EtOH (as negative control) for 16 h at 37°C (Huang *et al.,* 1996); cells were then harvested. The P–S protein was immunoprecipitated and labeled with ^{32}P as described above. For the treatment of EndoH or PNGaseF, the first anti-P6 immunoprecipitates were divided into two parts and boiled in 1× denaturation buffer (0.5% SDS, 1% β-mercaptoethanol) for 10 min followed by reacting with 50 units EndoH or PNGaseF (New England Biolabs, Beverly, MA) or without treatment at 37°C for 60 min in 1× G5 buffer (50 mM sodium citrate, pH 5.5) or 1× G7 buffer (50 mM sodium phosphate, pH 5.5) containing 1% NP-40 according to the manufacturer's manual. After being boiled, reaction mixtures were reprecipitated with anti-P6 antiserum.

RNA extraction and RT-PCR analysis

Total cellular RNA was isolated from transfected or untransfected cells by the Ultraspec-II RNA isolation kit (Biotecx, Houston, TX) as described in the manufacturer's manual. Five micrograms of total RNA was used for first-strand cDNA synthesis using oligo-dT as a primer. One-fiftieth of the cDNA products was subjected to polymerase chain reaction using P1 (5′-CCCCTATCCTATCAACACTT-3′, nts 2310 to 2329) and the antisense P2 (5′-CAATCCTCGAGAAGATTGAC-3′, nts 120 to 139) as primers and Taq DNA polymerase (Boehringer Mannheim, Germany) for 35 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min in a Perkin–Elmer thermocycler 9600. The 0.6-kb PCR products were purified and cloned into the pT-Adv vector (Clontech, Palo Alto, CA), according to the instructions of the manufacturer. The sequences were determined by the dideoxy termination method (Sanger *et al.,* 1977).

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