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Enhanced Replication of a Hepatitis B Virus Mutant Associated with an Epidemic of Fulminant Hepatitis

KIYOSHI HASEGAWA, JIAKANG HUANG, STEVEN A. ROGERS, HUBERT E. BLUM, AND T. JAKE LIANG*

Gastrointestinal Unit, Medical Services, and Molecular Hepatology Laboratory, MGH Cancer Center, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts

Received 25 October 1993/Accepted 9 December 1993

Hepatitis B virus (HBV) mutants unable to synthesize HBV e antigen have been described in association with fulminant hepatitis. We have cloned and sequenced the entire viral genome of an HBV strain associated with an epidemic of fulminant hepatitis. This strain contained, in addition to two G-to-A mutations in the precore region (nucleotides 1898 and 1901), numerous other mutations in conserved nucleotide positions resulting in significant amino acid substitutions in HBV gene products. We introduced either or both of the two G-to-A mutations into wild-type HBV by oligonucleotide-directed mutagenesis and generated replication-competent constructs of these mutants as well as the fulminant strain. Viral antigen synthesis, transcription, and replication were analyzed after transfection into human hepatoma cells. All viral constructs produced and secreted similar levels of envelope proteins (HBV surface antigen). Analysis of cellular lysate for core-specific immunoreactivity demonstrated a much higher level of core-associated antigens in cells transfected with the fulminant strain. While cells transfected with mutant and wild-type HBV DNAs synthesized similar levels of viral RNAs, the fulminant strain directed the synthesis of a much higher level of core-associated replicative intermediates (as well as virion particles) than the wild type and mutants with either or both of the precore mutations. Increase in the encapsidation of pregenomic RNA into core particles is likely the basis for the enhanced replication associated with the fulminant strain. Our study suggests that an HBV mutant with enhanced viral replication may be important in the pathogenesis of fulminant hepatic failure, and mutations other than the precore mutations may be responsible for this variant behavior.

Infection with hepatitis B virus (HBV) leads to a wide spectrum of liver injuries ranging from acute self-limited infection and fulminant hepatitis to chronic hepatitis with progression to cirrhosis and liver failure as well as to an asymptomatic chronic carrier state. There is accumulating evidence that HBV mutants unable to synthesize HBV e antigen (HBeAg) may influence the course of infection and clinical manifestations of disease (8, 10, 35). Many mutations inactivating the precore reading frame which is necessary for the synthesis of HBeAg have been described (7, 11). These mutations have been observed to arise de novo in patients chronically infected with wild-type HBV (38). Acute infection with some of these mutants appears to be associated with fulminant hepatitis (28, 31, 39). The pathogenesis of fulminant hepatitis B has been attributed to enhanced immune response against viral antigens (3, 47).

We have previously described the association of an HBV variant containing two precore mutations with an epidemic of fulminant hepatitis B (31). This nosocomial outbreak of five cases was remarkable for an unusually severe course of acute HBV infection leading to 100% mortality within a few days from hepatic and renal failure. The HBV strain responsible for this epidemic contained two G-to-A mutations in the precore open reading frame, one of them resulting in a stop codon. In this study, we cloned and sequenced the entire genome of this HBV strain and examined the viral antigen synthesis, transcription, and replication of this viral genome in transfected human hepatoma cells. In order to assess the biological effects of the two G-to-A mutations in the precore region, we introduced the mutations either separately or together into a wild-type HBV strain by oligonucleotide-directed mutagenesis and studied the biological properties of these mutants in vitro.

* Corresponding author. Mailing address: Gastrointestinal Unit, Massachusetts General Hospital, Jackson 812, Fruit St., Boston, MA 02114. Phone: (617) 726-3766. Fax: (617) 726-3673.

MATERIALS AND METHODS

HBV constructs. Serum DNA was purified from one of the patients with fulminant hepatitis B (patient 2) in the epidemic described previously (31). Three overlapping fragments of the HBV genome were generated by PCR amplification with three sets of HBV primers. The three primer sets are: 5′ ATTG CACCTGTATTCCCATCCCATC 3′ (primer 1, sense, nucleotides [nt] 593 to 617) and 5′ CGAGTCCAAGAGTCCTCT TAT (primer 2, antisense, nt 1656 to 1676), 5′ TCTTTCGG AGTGTGGATTCGCACTC 3′ (primer 3, sense, nt 2263 to 2287) and 5′ TAGGGTTTAAATGTATACCCA 3′ (primer 4, antisense, nt 823 to 843), and 5′ TGGAATTCGCATGGA GACCA CCGTGAAC 3′ (primer 5, sense, nt 1607 to 1627, with the underlined sequence indicating an *Eco*RI site) and 5′ TCTGCGACGCGGCGATTGAGA 3′ (primer 6, antisense, nt 2410 to 2430). The first PCR fragment was cloned into pGEM-5Zf(+) (Promega, Madison, Wis.), with an internal HBV restriction site of *Spe*I (nt 681) at one end and blunt-end ligation into the *Hin*cII site of the vector at the other end. The second fragment was cloned into pGEM-7Zf(+) with the internal HBV site of *Hin*dIII at one end (nt 2294) and blunt-end ligation into the *Sma*I site of the vector at the other end. The third fragment was generated by ligation of an internal HBV *Hin*dIII site (nt 2294) at one end and an *Eco*RI restriction site carried by primer 6 at the other end into pGEM-7Zf(+). A complete HBV genome was generated in two steps. First, fragments 1 and 2 were joined at the *Spe*I site

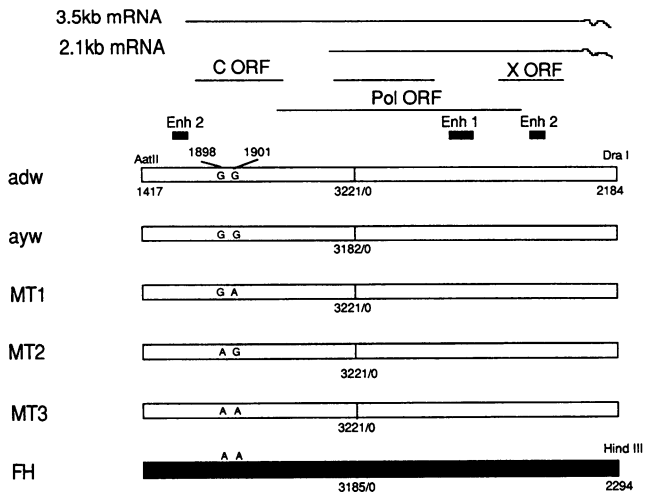

FIG. 1. Terminally redundant constructs of HBV mutants. The relative positions of RNA transcripts, open reading frames (ORFs) and regulatory elements (enhancers [Enh] 1 and 2) are displayed in reference to the genomic map of HBV (top). The various constructs with terminal redundance are shown below. Nucleotide sequences at positions 1898 and 1901 are shown for each construct. Mutant 1 (MT1), mutant 2 (MT2), and mutant 3 (MT3) contain a G-to-A mutation(s) at nt 1898, nt 1901, and both positions, respectively. The fulminant hepatitis (FH) construct is depicted in black. Pol, polymerase.

(nt 681) and ligated into the *Hin*dIII and *Sac*I sites of pGEM-7Zf(+). Second, a plasmid carrying fragments 1 and 2 was digested with *Sal*I [site in the carryover pGEM-5Zf(+) sequences], treated with exonuclease III (ExoIII) for limited 5′-to-3′ exonuclease digestion, and then redigested with *Hin*dIII after ExoIII was inactivated. A similar approach was applied to a plasmid carrying fragment 3 with the exception of *Eco*RI instead of *Sal*I as the first restriction enzyme used for digestion. Both fragments were purified and ligated to pGEM-7Zf(+) which had been subjected to *Hin*dIII restriction and calf intestinal phosphatase treatment. The ligation mixture was then treated with T4 polymerase in the presence of deoxynucleotides to eliminate any mismatched base pairs at the end of the complementary strand of each fragment which contains some vector sequences. The resulting clone carrying the full-length HBV genome was sequenced at the junction of fragments 1 and 3 to insure the preservation of the original DNA sequences.

Terminally redundant genomes (1.2 times the genomic length) of two wild-type HBV strains were constructed. The construct containing an adw subtype which is replication competent after transfection into HuH-7 cells has been described previously (5). The other was derived from an ayw subtype (21) which was a generous gift of H. Schaller (26, 43) and was constructed the same way as that of the adw subtype. A similar construct of the fulminant HBV strain (FH) with 1.2 times the genomic length was generated. While all the HBV constructs have the *Aat*II site (nt 1417) at the 5′ end, the 3′ end of the FH construct was at nt 2294 (*Hin*dIII), as opposed to nt 2184 (*Dra*I) in all other constructs. Since there was no *Dra*I site at nt 2184 in the FH genome, the nearest restriction site, *Hin*dIII, was used instead. A schematic diagram of the constructs is shown in Fig. 1.

Oligonucleotide mutagenesis. The Altered Sites in vitro mutagenesis system from Promega was used to generate three mutant HBV genomes with the precore mutations: one with a G-to-A mutation at nucleotide 1898 (MT1), the second with a G-to-A mutation at nucleotide position 1901 (MT2), and the third with both of the G-to-A mutations (MT3). The three mutant oligonucleotides are: 5′ GGGTGGCTTTAGGGCAT GGACATT 3′ (sense, nt 1888 to 1911), 5′ GGGTGGCTTT GGGACATGGACATT 3′ (sense, nt 1888 to 1911), and 5′ GGGTGGCTTTAGGACATGGACATT 3′ (sense, nt 1888 to 1911). Wild-type adw DNA was used as the template for mutagenesis. The mutations were confirmed by dideoxynucleotide sequencing. The same replication-competent genome as for the adw strain was constructed of each of the site-directed mutants.

Tissue culture and transfection. Human hepatoma HuH-7 cells were maintained in plastic dishes containing modified Eagle's medium (GIBCO, Grand Island, N.Y.) plus 10% fetal bovine serum at 37°C and 5% CO_2 (5, 6). HuH-7 cells were grown to 70% confluence and transfected with DNA by a modified $CaPO_4$ transfection method (5, 6).

Viral protein, RNA and DNA analyses. HBV surface antigen (HBsAg) and HBeAg were determined by commercially available radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) (Centocor, Malvern, Pa., and Nippon Pharmaceuticals, respectively). For the detection of hepatitis B core antigen (HBcAg), cell lysate was incubated with polystyrene beads coated with a rabbit polyclonal HBcAg-specific antibody (Dako, Carpinteria, Calif.). Horseradish peroxidase-labelled anti-core antibody from a Corzyme kit (Abbott Laboratories, North Chicago, Ill.) was added as the detecting antibody after the beads were washed several times. The pTKGH plasmid containing the human growth hormone (hGH) gene driven by the thymidine kinase enhancer and promoter was cotransfected with various HBV constructs to control for transfection efficiency, and secreted hGH in medium was measured with a radioimmunoassay kit from Nichols Institute Diagnostics (San Juan Capistrano, Calif.). Typically, 15 μg of HBV constructs was cotransfected with 1 μg of pTKGH plasmid into HuH-7 cells grown in 10-cm-diameter culture dishes. For metabolic labelling, cells were labelled with 250 μCi of [^{35}S]methionine and [^{35}S]cysteine (Dupont, Wilmington, Del.) per ml for 2 h in methionine- and cysteine-free medium 3 days after transfection. Cell lysates were prepared and subjected to immunoprecipitation as described previously (6). For immunoprecipitation of HBV envelope proteins, two monoclonal antibodies (5D3 and 5C11) specific for HBsAg (51) were used; for HBcAg, a rabbit polyclonal antiserum specific for core antigen (Dako) was employed.

Three to four days after transfection, HuH-7 cells were harvested for viral DNA and RNA analysis. RNA was prepared by the guanidium isothiocyanate-acid-phenol method (17) and analyzed by formaldehyde agarose gel electrophoresis and hybridized with an HBV-specific probe. Viral replicative intermediates associated with intracellular core particles were isolated by ultracentrifugation of cell lysate through a 30% sucrose cushion and then analyzed by Southern blot hybridization (5, 6). Endogenous polymerase activity of the isolated core particles was analyzed as described previously (43). Secreted viral particles were immunopurified by using anti-HBs(5D3)-coupled Sepharose (32). The washed beads were then subjected directly to endogenous polymerase activity analysis (43). Secreted virions were first ultracentrifuged to form a pellet and then purified through a CsCl isopycnic centrifugation gradient (5, 6). Fractions containing HBsAg immunoreactivity were pooled, and viral nucleic acids were extracted and analyzed by Southern blot hybridization.

Analysis of core-associated RNA. Three days after the

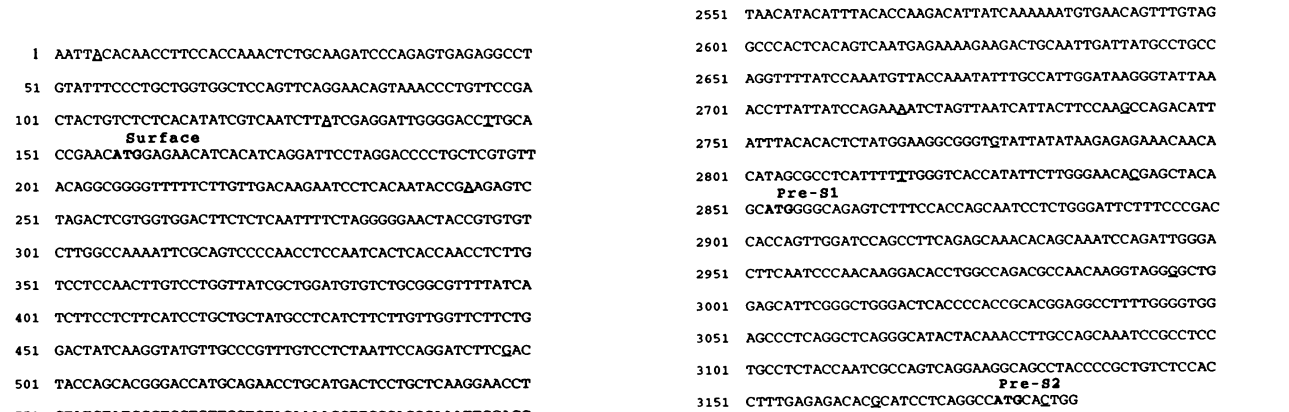

FIG. 2. Complete nucleotide sequence of the FH strain. Underlined nucleotides indicate mutations at conserved positions. Boldface nucleotide sequences represent start (ATG) and stop codons in each of the open reading frames. The beginning of each open reading frame is indicated above the start codon. RT/Pol, reverse transcriptase/polymerase.

transfection of HuH-7 cells with various HBV constructs, cells were lysed with RNA extraction buffer containing 10 mM Tris-HCl (pH 8.6), 0.14 M NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Nonidet P-40, and RNAsin (500 U/ml; Promega) on ice. After centrifugation to form a pellet of the nuclei, 10% of the supernatant was subjected to RNA isolation by the guanidinium isothiocyanate-acid-phenol method (17). The remaining supernatant was subjected to ultracentrifugation to purify the core particles as described above. The pellet containing the core particles was treated with *Staphylococcus aureus* nuclease (Boehringer Mannheim, Indianapolis, Ind.) at a concentration of 8 μg/ml for 60 min at 37°C to eliminate any contaminating RNA and DNA. Core-associated RNA was then isolated. For primer extension analysis, an HBV primer (5′ TCTAAGGCTTCTCGATACAGAGCTG 3′) spanning nt 2006 to 2030 in the antisense orientation was end labelled with [γ-^{32}P]ATP and then reacted with isolated RNA by a standard protocol (1). The products were analyzed on a 6% polyacrylamide-urea gel.

Nucleotide sequence accession number. The HBV nucleotide sequence has been entered into GenBank under accession number L27106.

RESULTS

Using three sets of PCR primers, we cloned the HBV genome from one of the patients with fulminant hepatitis B. The complete nucleotide sequence of this genome was obtained and is shown in Fig. 2. The genome is 3,185 bp long and has a genetic organization identical to those of known HBV strains. The sequence was compared with all known HBV sequences published previously (4, 14, 20, 21, 27, 36, 37, 40, 41, 45, 50) and was most similar to that of the ayw 2 strain (4). Numerous mutations affecting sequences conserved in other HBV strains were detected (Table 1). These mutations were not a result of PCR artifacts and had been confirmed by sequencing of at least two other independent PCR clones. Other mutations were also detected during sequence analysis of these clones; they are probably not important, because they either affect nonconserved sequences or represent viral heterogeneity. An insertion of a GCC triplet after nt 1630 and 43 mutations (underlined in Fig. 2) affecting conserved nucleotide

TABLE 1. Conserved amino acid substitutions in the FH strain[a]

Region	Position	Mutation
Precore/core	28	Trp–Stop
	29	Gly–Asp
	57	Val–Ala
	68	Tyr–Phe
	94	Glu–Asp
	95	Leu–Val
	115	Val–Ile
	126	Lys–Arg
	135	Ile–Leu
	160	Pro–Gln
	198	Ser–Pro
	210	Glu–Gln
Pre-S/S	110	Gln–His
	113	Ser–Tyr
	160	Pro–Leu
	193	Gln–Lys
RT/Pol	45	Asn–Asp
	144	Thr–Ala
	156	Ile–Val
	169	Cys–Phe
	177	Gln–His
	229	Ser–Gly
	285	Ser–Ala
	291	Val–Leu
	293	Phe–Leu
	330	Leu–His
	335	Leu–Phe
	342	Ala–Thr
	458	Asn–Asp
	644	Met–Lys
	704	Met–Val
X	85	Insert of Ala
	94	His–Tyr
	127	Glu–Asp
	128	Ile–Asn
	132	Val–Ile
	133	Phe–Tyr

[a] Amino acid positions are numbered from the start codon of each protein. The first amino acid given for each position represents the wild-type sequence, and the second indicates the mutation in the FH strain. These mutations have been confirmed by sequencing of independent PCR clones. RT/Pol, reverse transcriptase/polymerase; S, surface.

positions, 37 of which resulted in amino acid substitutions, were noted. Mutations were identified in all open reading frames: 12 in the precore/core region, 15 in the reverse transcriptase/polymerase region, 4 in the pre-S/surface region, and 6 in the X region (Table 1).

A replication-competent construct of the mutant HBV genome was generated. Constructs of two distinct wild-type HBV strains, one adw and the other ayw subtype, were used as wild-type controls. Three HBV mutants with either or both G-to-A substitutions in the precore region (nt 1898 and 1901) generated by oligonucleotide-directed mutagenesis of the wild-type adw strain were constructed. All the HBV constructs contained 1.2 times the length of the HBV genome and spanned nt 1417 (*Aat*II) to 2294 (*Hin*dIII) for the FH strain or to 2184 (*Dra*I) for the others (Fig. 1).

The HBV constructs were transfected into HuH-7 cells and assayed for the production of HBsAg and HBeAg. The results are shown in Table 2. An hGH gene-containing plasmid (pTKGH) was cotransfected with each HBV construct to control for the transfection efficiency. The levels of HBsAg

TABLE 2. HBsAg, HBeAg, and HBcAg production in cells transfected with HBV constructs[a]

Construct	Production of antigen in:				
	Culture medium			Cell lysate (OD)	
	hGH (ng/ml)	HBsAg (S/N)	HBeAg (OD)	HBeAg	HBcAg
Control	5.4	1	0.01	0.05	0.02
adw	7.8	176	0.676	0.292	0.047
ayw	9.8	112	1.041	0.498	0.057
MT1	5.8	131	0.698	0.198	0.058
MT2	9.4	195	0.014	0.029	0.046
MT3	7.7	200	0.011	0.031	0.045
FH	8.6	151	0.002	0.015	0.514

[a] HuH-7 cells were maintained and transfected with various HBV constructs. On day 4, culture media were harvested for detection of HBsAg and HBeAg and cells were harvested for HBeAg and HBcAg determination as described in Materials and Methods. Individual values from HBsAg radioimmunoassay are shown (a signal/noise [S/N] ratio of greater than 2 is considered positive). Titers of HBeAg or HBcAg are displayed as optical densities (OD) from ELISA. Transfection efficiency was controlled for by cotransfection with pTKGH. Results are representative of at least three separate experiments.

detected by radioimmunoassay either in cell lysate (data not shown) or secreted in the culture medium (Table 2) were within a twofold variation among cells transfected with various HBV constructs. The minor variations in HBsAg production observed in this experiment were not significant, since they were not reproducible in other experiments. Since large envelope protein is important in the maturation of core particles and eventual morphogenesis to infectious virions (9, 48, 49), metabolic labelling and immunoprecipitation were used to examine the synthesis of all three forms of envelope proteins. The results are shown in Fig. 3. With controls for transfection efficiency, all the HBV constructs produced similar levels of

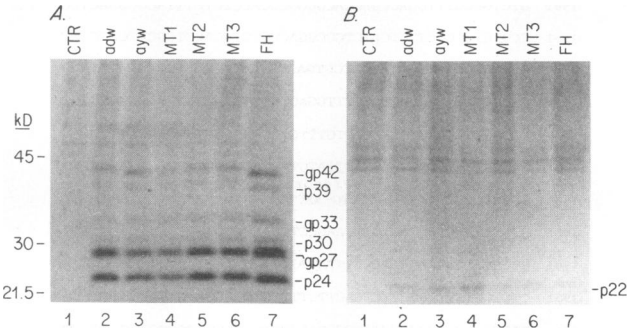

FIG. 3. Synthesis of HBV envelope and core proteins in cells transfected with HBV constructs. HuH-7 cells were grown, transfected, labelled metabolically on day 3, and treated as described in Materials and Methods. Cell lysates were subjected to immunoprecipitation with either HBsAg- or HBcAg-specific antibodies, washed, and electrophoresed by SDS-PAGE. Autoradiography was performed after the gel was fixed and treated with Amplify (Dupont). (A) HBsAg synthesis in various HBV constructs. Six forms of HBsAg are seen: p24 and gp27, p30 and gp33, and p39 and gp42 are large envelope proteins, with gp representing the glycosylated form of each envelope protein. (B) HBcAg production. p22 is the only species detected in the cell lysate. Lanes contain transfections of the indicated DNAs. CTR, control transfection. Cotransfection of pTKGH plasmid demonstrated similar levels of transfection efficiency, with the exception of FH-transfected cells, which produced approximately two times as much hGH as the others did in this experiment.

large, middle, and major envelope proteins by immunoprecipitation. The large envelope protein produced by the HBV adw constructs migrated slightly more slowly than those of the ayw and FH strains. This finding is consistent with the larger pre-S1 open reading frame in the adw strains. Since the HBeAg kit from Abbott Laboratories detects HBeAg as well as HBcAg, and HBcAg can be detected in a culture medium of cells transfected with HBV DNA, we used an HBeAg-specific ELISA kit to measure the accumulation of HBeAg in cell lysate and culture medium (Table 2). The two HBeAg-specific monoclonal antibodies used in this assay have been described previously (24). Similar levels of HBeAg were detected in the two wild-type HBV strains and the MT1 mutant. No appreciable HBeAg was detected in cells transfected by constructs FH, MT2, and MT3.

Using a core antigen-specific ELISA system, we detected a significantly higher level of core antigen immunoreactivity in a lysate of cells transfected with the fulminant strain. In order to distinguish the possibilities of increased synthesis versus decreased turnover, metabolic labelling followed by immunoprecipitation using core-specific antibodies was performed. The results demonstrated that the rate of core antigen synthesis (p22) was similar among all the HBV constructs (Fig. 3). Furthermore, a pulse-chase experiment was performed to evaluate the turnover rate of core antigens. After metabolic labelling with [^{35}S]methionine and [^{35}S]cysteine for 3 h, cells transfected with the wild-type ayw and FH HBV DNAs were harvested at 0, 4, 8, and 12 h and subjected to immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 4). The p22 core signal appears to be higher at time zero in the FH-transfected cells than that in the wild-type-transfected cells (absolute optical density of 6.098 versus 3.334 by densitometry). This discrepancy can be explained by a higher transfection efficiency in the FH-transfected cells in this experiment (about twice the hGH activities of the wild-type-transfected cells). Figure 4B illustrates the turnover of core proteins versus time by densitometric measurement of the autoradiogram. The results indicated that the newly synthesized FH cores appear to have a longer (about two times) intracellular half-life than the wild-type cores. These observations are consistent with our previous finding of a much higher level of core immunoreactivities in cells transfected with the FH strain than those transfected with other HBV strains. Cellular RNA was purified from each of the transfections and analyzed on Northern (RNA) gels for the synthesis of HBV transcripts (Fig. 5). Two species of HBV RNA, 3.5 and 2.4 to 2.1 kb, were present at similar levels in all the transfected cells after correction for transfection efficiency and the amounts of RNA loaded on the gel.

HBV replication was then analyzed by Southern blot analysis of the replicative intermediates in the cell lysates (Fig. 6A). The results showed clearly that the FH construct directed a much higher level of replicative intermediates than the wild-type HBV. All the site-directed mutants produced wild-type levels of replicative intermediates. Analysis of endogenous polymerase activity of purified core particles revealed similar findings (Fig. 6B). These experiments have been repeated multiple times, and the results described above were consistently reproducible.

In order to demonstrate that Dane particle (matured virion) production was also increased in cells transfected with the FH strain, we analyzed secreted viral particles in culture medium. HBsAg-associated viral particles were immunopurified and subjected to endogenous polymerase activity analysis. Using a sufficient amount of anti-HBs-coupled Sepharose beads, we

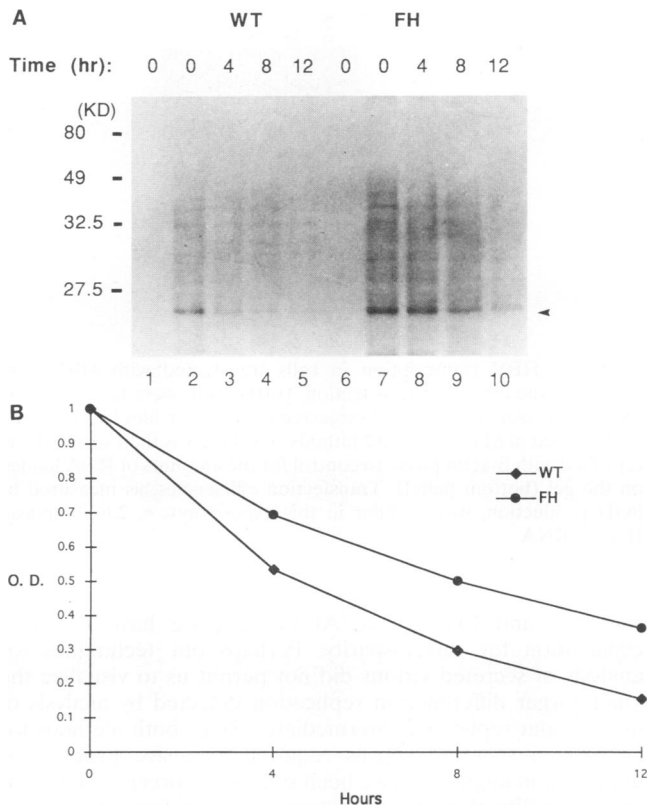

FIG. 4. Half-life of cores in cells transfected with HBV constructs. HuH-7 cells were grown, transfected, and labelled metabolically on day 3 for 2 h. (A) At 0, 4, 8, and 12 h, one set of transfected cells was lysed and subjected to immunoprecipitation with HBcAg-specific antibodies, washed, and electrophoresed by SDS-PAGE as described previously. Lanes 1 to 5 represent cells transfected with the wild type (WT), and lanes 6 to 10 represent cells transfected with the FH mutant. Lanes 1 and 6, cells collected at 0 h and treated with preimmune serum; lanes 2 and 7, cell lysates harvested at 0 h and immunoprecipitated with HBcAg-specific antibodies; lanes 3 and 8, at 4 h; lanes 4 and 9, at 8 h; lanes 5 and 10, at 12 h. The arrowhead indicates p22. (B) Signal intensities of core proteins in each sample quantitated by densitometry (Molecular Dynamics) and adjusted to the optical density (OD) reading of time zero that was standardized to 1. The adjusted OD readings were then plotted versus time.

were able to immunopurify HBsAg-associated particles quantitatively from the culture medium, as demonstrated by a marked reduction of HBsAg titer (<5% of the original titer) in the culture medium supernatants (not shown). As shown in Fig. 7A, enhanced endogenous polymerase activity was evident in cells transfected with the fulminant strain (at least five times more by densitometry than the others). This difference was also reproducible in other experiments. The minor variations seen among the other samples were not borne out in other experiments. In a parallel experiment, secreted virions in culture medium were purified through CsCl gradients and viral DNAs were extracted and analyzed by Southern blot hybridization (Fig. 7B). As shown in Fig. 7B, a higher level of viral genomic DNA was present in the culture medium of cells transfected with the fulminant strain. These data indicate that enhanced intracellular viral replication is also accompanied by an increase in virion secretion in FH-transfected cells. However, the difference in secreted virions was not as dramatic as the difference in cellular replicative intermediates between the

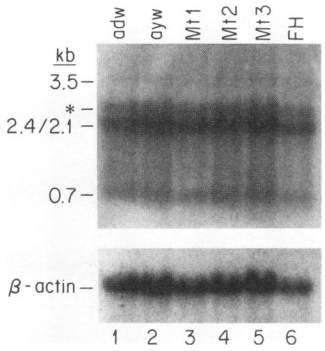

FIG. 5. HBV transcription in cells transfected with HBV constructs. Three days after transfection, HuH-7 cells were harvested and their RNAs were isolated and subjected to Northern blot hybridization as described in Materials and Methods. The blot was then stripped and reprobed with β-actin probe to control for the amounts of RNA loaded on the gel (bottom panel). Transfection efficiencies, as measured by hGH production, were similar in this experiment. *, 2.6-kb spliced HBV mRNA.

wild-type and FH strains. At present, we have no good explanation for this disparity. Perhaps our techniques for analysis of secreted virions did not permit us to visualize the much larger difference in replication detected by analysis of intracellular replicative intermediates. Since both methods for analysis of secreted virions required extensive procedures, quantitation might not have been sufficiently precise. However, the possibility that the FH cores could be less efficient for envelopment cannot be eliminated at present.

Increased HBV replication can be attributed to enhancement at any one of the various stages of the viral life cycle. The data presented so far have indicated an increase in all forms of replicative intermediates, from single-stranded DNA to partially double-stranded circular genomes. We surmise that the step which results in enhanced viral replication of the FH mutant occurs before or during the reverse transcription of HBV pregenomic RNA to viral DNA. Since most of the encapsidated pregenomic RNA is likely to be converted to DNA by reverse transcriptase/polymerase stoichiometrically

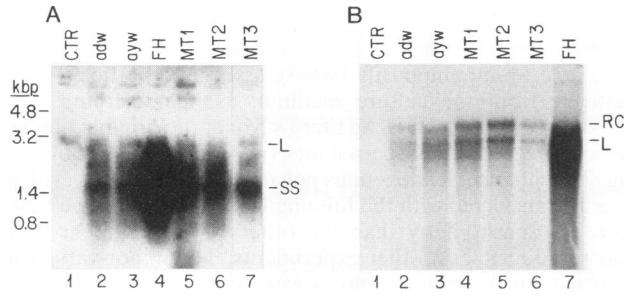

FIG. 6. HBV replication in cells transfected with HBV constructs. Four days after transfection, HuH-7 cells were harvested and core particle-associated HBV DNAs were isolated and subjected to Southern blot hybridization and endogenous polymerase assay as described in Materials and Methods. (A) Southern blot hybridization analysis of core particle-associated viral DNA. (B) Endogenous polymerase activity of purified viral core particles. Lane contents are as indicated. CTR, control transfection; SS, single-stranded HBV DNA as the replicative intermediate; L and RC, double-stranded linear and relaxed circular HBV genomes, respectively. Cotransfection of the pTKGH plasmid demonstrated similar levels of transfection efficiency.

within the core particle, we reason that the step of encapsidation of pregenomic RNA is likely to be involved. In order to study the level of viral encapsidation, we isolated intracellular core particles, purified the encapsidated pregenomic RNA, and analyzed its level by primer extension analysis (Fig. 8). Analysis of total RNA in cells transfected with various HBV constructs revealed three species of primer extension products, whereas only one major species was detected in reactions with the core-associated RNA. These observations are consistent with previous reports demonstrating that three species of mRNA with variable 5′ ends are synthesized but only the shortest one is encapsidated (30, 52). On one hand, the signals of primer-extended products of total RNA were comparable among all the HBV constructs, except that of the FH strain, which was weaker than the others. This difference was accounted for by a lower transfection efficiency of the FH-transfected cells in this experiment (approximately half as much hGH was produced). On the other hand, the signal generated by the core RNA in the FH-transfected cells was much more intense than the others, suggesting a much higher level of encapsidation of pregenomic RNA. Several smaller but significant species of extended products were seen preferentially in the FH-transfected samples. These probably represent strong stops of the primer extension reaction. In addition, we analyzed the total and core RNAs by RNase protection experiments. The results were comparable to those of the primer extension analysis and also demonstrated higher levels of encapsidated pregenomic RNA (data not shown).

DISCUSSION

In this study, we cloned and sequenced the entire viral genome of an HBV strain associated with an epidemic of fulminant hepatitis. Comparisons with all known published HBV sequences revealed mutations occurring at numerous conserved nucleotide positions. Multiple amino acid substitutions at conserved residues were present in all four open reading frames. It is interesting that mutations tended to cluster in the precore/core protein (Table 1). Since core antigen has been postulated as the target of cell-mediated immunity (35), it is tempting to speculate that these core mutations might contribute to a more severe course of disease, as suggested by a recent publication (19). Two G-to-A mutations (nt 1898 and 1901) in the precore open reading frame were introduced into a wild-type HBV strain by oligonucleotide-directed mutagenesis. Replication-competent genomes of the site-directed mutants as well as the fulminant viral strain were constructed and transfected into HuH-7 human hepatoma cells. Analysis of cellular lysate for core-specific immunoreactivity demonstrated a much higher level of core-associated antigens in cells transfected with the fulminant strain. Cells transfected with mutant and wild-type HBV DNAs produced comparable levels of viral RNAs. The HBV mutants with either or both of these two precore mutations synthesized similar levels of replicative intermediates. In contrast, the fulminant viral DNA was capable of directing the synthesis of a much higher level of core-associated replicative intermediates upon transfection into human hepatoma cells. This result is consistent with the above finding of a higher level of core-associated immunoreactivity in cells transfected with the fulminant strain.

Since only a minor fraction of the core antigens synthesized are assembled into complete virions, the majority of core antigens are probably either degraded intracellularly or released into medium as partially assembled, empty cores (30a). Our data suggest that more core antigens produced by the FH

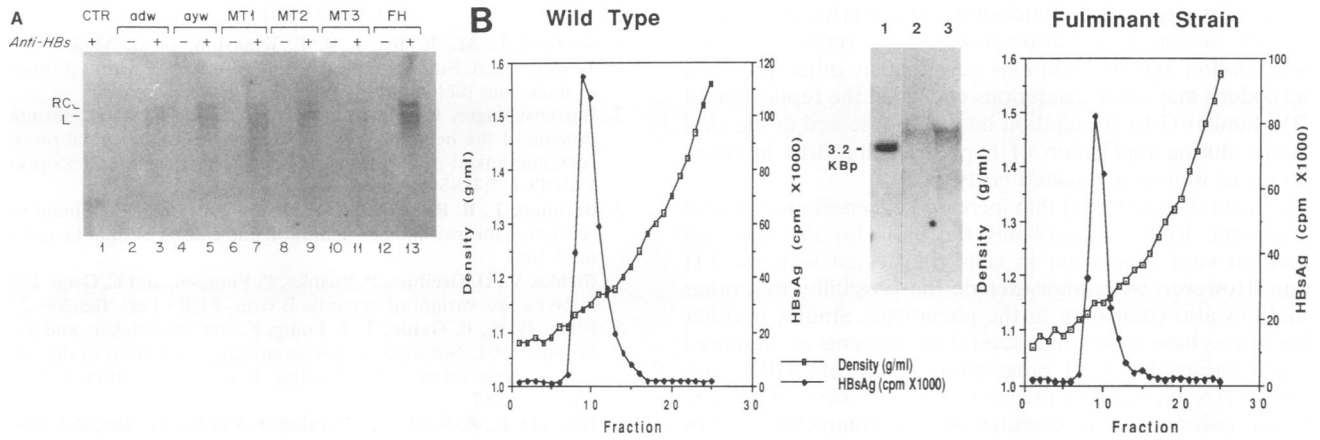

FIG. 7. Virion secretion in culture medium. Five days after transfection, spent culture media were collected and HBsAg-containing viral particles were isolated and subjected to an endogenous polymerase assay as described in Materials and Methods. (A) Lane 1, control transfection (CTR); lanes 2 and 3, adw strain; lanes 4 and 5, ayw strain; lanes 6 and 7, MT1; lanes 8 and 9, MT2; lanes 10 and 11, MT3; lanes 12 and 13, FH. Lanes 1, 3, 5, 7, 9, 11, and 13 show reactions with anti-HBs-coupled Sepharose beads (anti-HBs); lanes 2, 4, 6, 8, 10, and 12 represent reactions with Sepharose beads coupled to an irrelevant antibody (anti-human chorionic gonadotropin). L and RC, double-stranded linear and relaxed circular HBV genomes, respectively. (B) In a parallel experiment, secreted virions were first ultracentrifuged to form a pellet and then purified through CsCl equilibrium density gradients. The density and HBsAg content of the gradient are shown. Fractions 8 to 16 containing HBsAg immunoreactivity (buoyant densities between 1.14 and 1.25 g/ml) were pooled, and viral nucleic acids were extracted and analyzed by Southern blot hybridization (inserted panel). Fraction 17 from each gradient did not contain any hybridizable HBV sequences (not shown), indicating that all the virion DNAs were included in the pooled fractions. Lane 1, molecular marker; lane 2, ayw strain; lane 3, FH. Transfection efficiencies, as measured by hGH production, were similar in this experiment.

strain are being selectively retained in the cells, most likely for the assembly of complete core particles as replicative intermediates in the formation of mature virions. Enhanced virion secretion was also evident in FH-transfected cells. These observations, in toto, suggest a much-enhanced viral replication associated with this fulminant HBV strain. However, there is a discrepancy between the levels of increase of the intracellular replicative intermediates and secreted virions in cells transfected with the FH mutant. At present, we do not have a good explanation for it.

Regarding the significance of the two precore mutations (nt 1896 and 1901), it is conceivable that they alter the regulation of HBV transcription and replication by providing a thermodynamically stable stem-loop structure that was proposed to be the packaging signal for the encapsidation of HBV pregenomic RNA (22, 26), resulting in an increase of -3.9 kcal/mol (-16.3 kJ/mol) in Gibbs free energy (46). In this study, we demonstrated that neither of these two mutations alone nor both together altered viral protein synthesis, transcription, or replication in transfected human hepatoma cells. Since these two precore mutations occur in the pregenomic RNA twice, it is necessary to exclude the possibility that mutations at these positions in the 3′ end might be important. In order to address this issue, we generated three additional constructs with either or both mutations in both ends of the replication-competent genome and showed that these constructs were similar to their counterparts (MT1, MT2, and MT3) in transfection experiments (data not shown). Furthermore, in order to eliminate the possibility that the slightly longer construct of strain FH (110 bp longer than the other constructs at the 3′ end) might be responsible for the enhanced replication, we generated two other terminally redundant constructs with their 3′ ends at the *Bgl*II site (nt 1984), one containing the ayw strain and the other containing the FH strain. The FH construct again demonstrated a much higher level of replication than the ayw construct (data not shown).

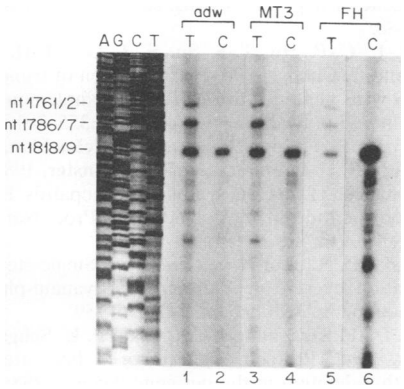

FIG. 8. Primer extension analysis of encapsidated viral RNA. Three days after transfection, total and core-associated RNAs (indicated by T and C, respectively, above the numbered lanes) were purified and subjected to primer extension analysis as described in Materials and Methods. Primer extension products were electrophoresed on a 6% polyacrylamide-urea gel and autoradiography using an intensifying screen with exposure time of 24 h at -80°C was performed. A dideoxy-sequencing reaction using the same primer on the adw strain is shown on the left (lanes marked A, G, C, and T). Lanes 1, 3, and 5, reactions with total RNA; lanes 2, 4, and 6, the core RNA.

At present, we do not know whether these two precore mutations affect the life cycle of HBV in vivo. A woodchuck hepatitis virus mutant containing the same first precore mutation appeared to replicate normally in woodchucks (12). Therefore, the absence of HBeAg production conferring a biological advantage during immune selection in infected hosts may indeed be the major reason for the emergence of these HBV precore mutants. Within the precore open reading frame, there are several possible codons which can be mutated to a stop codon resulting in the abrogation of HBeAg synthesis. Why the mutation occurs predominantly in the codon involving nt 1898 is not apparent. This could be explained by preferential

mutagenesis targeting this nucleotide during HBV replication. Since the precore region also contains direct repeat 1 and the encapsidation signal, mutations resulting in other potential stop codons may exert deleterious effects on the replication of HBV. Similar G-to-A mutation has been observed in the viral genome during replication of hepatitis delta virus in tissue cultures as well as in infected hosts (33).

Our data demonstrated that increased encapsidation of viral pregenomic RNA was probably the basis for the observed enhanced viral replication in cells transfected with the FH strain. However, we cannot exclude the possibility that other mutations also contribute to the phenotype. Studies in other laboratories have shown that several components are required for the encapsidation of pregenomic RNA into HBV core particles (15, 23, 30). The pregenomic RNA, the core protein, and the polymerase are essential for the completion of this process. By genetic analysis, the 5′ region of polymerase, i.e., the terminal protein, is the important element for interaction with other components (2, 15). However, a recent report described how a mutation in the 3′ region of the duck HBV polymerase appeared to prevent encapsidation of the duck HBV genome (13). A mutation(s) involving these components in the fulminant viral genome may contribute to a higher level of replicative intermediates observed in cells transfected with this viral DNA.

Although HBV is not thought to be cytopathic, it is conceivable that enhanced viral replication contributes to a more severe form of liver disease in several ways. First, during acute HBV infection, enhanced viral replication could result in an increased number of hepatocytes being infected, thus leading to more hepatocellular injury targeted by host immunocytes. Second, since core antigen has been proposed to be the target of the cell-mediated immune response (35), increased core antigen accumulation in infected hepatocytes may contribute to a more vigorous immune response against the cells. In addition, several lines of evidence have suggested that dysregulated HBV gene expression can induce a cytopathic effect (16, 42, 53). It is of interest that increased virulence of a viral strain has been attributed to enhanced viral replication in other viral systems as well (18, 25, 44). At present, we have no direct indication that enhanced HBV replication is indeed the pathogenetic basis of fulminant hepatitis. The only indirect evidence supporting this notion is that this FH strain was transmitted from one patient to five other individuals, all of whom developed fulminant hepatitis. However, we cannot exclude other viral and/or host factors as important in the pathogenesis of fulminant hepatitis. In addition, our studies were limited to transfection experiments in tissue culture, which clearly does not reflect in vivo pathogenesis of HBV infection. To address this issue further, we need to study more cases of fulminant hepatitis B and develop an in vivo model to assess the biological significance of mutations identified by the above approach. Other hepadnaviruses, such as woodchuck hepatitis virus, will undoubtedly provide valuable models to address some of these issues.

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