Hepatitis B Virus Precore Mutation and Fulminant Hepatitis in the United States

A Polymerase Chain Reaction–based Assay for the Detection of Specific Mutation

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Abstract

Hepatitis B virus (HBV) variants with precore mutation(s) resulting in the absence of HBeAg production have been associated with the occurrence of fulminant hepatitis in Japan, Israel, and southern Europe, where the prevalence of this HBV strain appears common. In areas such as United States, where HBV infection is not endemic, the role of this mutant virus in fulminant hepatitis is unknown. We developed an amplification refractory mutation detection system to detect specifically the presence of the G to A mutation at nucleotide position 1898, which is the most frequently observed mutation resulting in a precore stop codon. In addition, this method provided a quantitative measurement of the relative ratio of one strain to the other. Using this system, we tested HBV strains for the presence of the stop codon mutation in sera from 40 cases of fulminant hepatitis B occurring in the United States. Serum HBV DNAs from 28 patients were analyzed successfully. A mixture of wild-type and mutant strains in various ratios were observed in 15 patients, wild type exclusively in 11, and mutant exclusively in 2. Four of these patients had undergone liver transplantation for HBV-associated cirrhosis and developed fulminant HBV–associated hepatitis after transplantation. Pre- and posttransplant serum samples from one patient were analyzed: a mixture of wild-type and mutant HBV strains was detected in both samples. Our study demonstrated that both wild-type and mutant HBV strains are associated with fulminant hepatitis, and that in some patients in the United States, factors other than precore mutations contribute to the development of fulminant hepatitis. (*J. Clin. Invest.* 1994. 93:550–555.) Key words: hepatitis B virus • fulminant hepatitis • precore mutation • polymerase chain reaction • hepatitis C virus

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1. *Abbreviations used in this paper:* ARMDS, amplification refractory mutation detection system; HBV, hepatitis B virus; IVDA, intravenous drug addict; nt, nucleotide.

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Introduction

Infection with hepatitis B virus (HBV)[1] is associated with a broad spectrum of liver injury, ranging from acute self-limited infection to fulminant hepatitis, chronic hepatitis with or without progression to cirrhosis and liver failure, as well as to an asymptomatic chronic carrier state. Evidence has been accumulating to indicate that HBV mutants unable to synthesize HBeAg may influence the course of infection and clinical manifestations of disease (1–3). Many mutations inactivating the precore open reading frame necessary for the synthesis of HBeAg have been described (4, 5). These mutations have been observed to arise de novo in patients chronically infected with wild-type HBV (6), and acute infection with such mutants appears to be associated with fulminant hepatitis (7–9).

We have previously described the association of an HBV variant containing two precore mutations with an epidemic of fulminant hepatitis B in Israel (7). The HBV strain responsible for this epidemic contained two G to A mutations in the precore open reading frame (nucleotides [nt] 1898 and 1901), one of them resulting in a stop codon. Other laboratories have also reported similar findings in patients with sporadic fulminant hepatitis B in Japan (8, 9). Using an amplification refractory mutation detection system (ARMDS) (10) to detect specifically the presence of the G to A mutation at nt position 1898, we studied 40 cases of sporadic fulminant hepatitis B that had occurred in various regions of United States. The system is based on the absolute inability of oligonucleotides with mismatched 3′ residues to function as PCR primers under appropriate conditions.

Methods

Patients. Serum samples were collected from 40 patients with sporadic fulminant hepatitis B that had occurred in various regions of the United States: New England (seven patients), mid-Atlantic coast (19 patients), Colorado (two patients), Alabama (two patients), Florida (two patients), Washington (two patients), Alaska (three patients), and Texas (three patients). Most of the serum samples were collected at the Massachusetts General Hospital, Jefferson Medical College, the Baylor College of Medicine–affiliated hospitals, and Alaska Native Medical Center. The eight cases from Colorado, Alabama, Florida, and Washington were collected through an epidemiological surveillance program established by the Center for Disease Control and Prevention (Atlanta, GA). Since 1981, intensive surveillance of acute viral hepatitis has been conducted in four "sentinel counties" in the United States (Denver, CO; Jefferson, AL, Pinellas, FL; and Pierce, WA). 16 cases of acute, self-limited HBV hepatitis were included for comparative studies: four from New England; two from Denver, CO; one from Jefferson, AL; two from Pinellas, FL; one from Pierce, WA; and six from Texas.

Serum samples obtained during initial hospitalization within 6 wks from the onset of illness were stored frozen at −80°C except for the cases from CDC (Atlanta), where they were stored at −20°C. The clinical characteristics of 28 patients whose serum samples we were able to analyze successfully are summarized in Table I. All patients had circulating IgM anti-HBc, except for patients 20–23, who had end-stage HBV-associated cirrhosis, and in whom fulminant hepatitis B developed after transplantation. These four patients developed acute hepatic failure histologically and serologically consistent with fulminant hepatitis B, within 3 mo after transplant. Serum samples from the preoperative stage, as well as during the occurrence of fulminant hepatitis were available from one such patient (patient 20). For the other three patients, we were only able to obtain serum samples during the fulminant hepatitis phase.

Amplification refractory mutation detection system. Serum DNA was isolated as described previously (11). Extracted DNA was subjected to ARMDS for specific detection of the G to A mutation at nt 1898 (10). This system is depicted schematically in Fig. 1. Two HBV primers (primers 1 and 2) spanning nt 1605–2432 of the HBV genome were used in the first PCR amplification. An aliquot of reaction product was then amplified further in a second reaction with primers 3 and 4, which are contained within the region spanned by the first two primers. Primer 3 had either a G (primer 3W) or A (primer 3M) at the 3′ end, corresponding to nt position 1898. Therefore, primers 3W and 3M were referred to as the "detection primers," one specific for the wild-type G and the other for the mutant A sequence, respectively. In addition, a mutation of T to G was introduced at the third position from the 3′ end of the detection primer to further enhance the specificity of the amplification reaction (10). The primer sequences were: primer 1, 5′ GTTGCATGGAGACCACCGTGAAC 3′ (sense, nt 1605–1627); primer 2, 5′ GCTTCTGCGACGCGGCGATTGAGA 3′ (antisense, nt 2432–2410); primer 4, 5′ CGAGGGAGTT CTTCTT-CTAG 3′ (antisense, nt 2394–2375). The sequences for primers 3W and 3M are shown in Fig. 1. The first PCR amplification continued for 35 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 2.5 min), and the second PCR was designed for 30 cycles with the annealing step at 62°C for 1 min and the elongation step at 72°C for 1.5 min. The reaction volume was 25 μl in each of the PCR reactions, and the standard PCR reaction was performed for the first PCR amplification. 10 μl of the first PCR reaction was subjected to Southern blot hybridization with a ^{32}P-HBV-specific probe. The samples with a positive PCR signal were subjected to a second PCR amplification with either wild-type or mutant-specific primers. Then 1 μl of the first PCR product was amplified with standard PCR reagents, except for the addition of 5 pmol of each primer (half of the standard concentration) and the inclusion of 1 U of Perfect Match (Perkin-Elmer Cetus Corp. Norwalk, CT) per 100 μl of PCR reaction mixture. This condition was determined experimentally to optimize the ARMDS method for specific detection of the G to A mutation at nt position 1898.

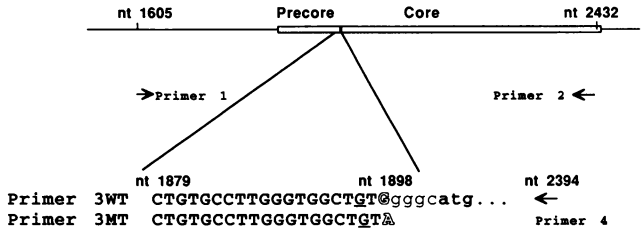

Figure 1. Amplification refractory mutation detection system. Two HBV primers (primers 1 and 2) spanning nt 1605–2432 of HBV genome were used in the first PCR amplification. An aliquot of reaction product was then further amplified in a second reaction with primers 3 and 4 contained within the region spanned by the first two primers. Primer 3 has either an G (primer 3W) or A (primer 3M) at the 3′ end (*outlined*), corresponding to nt position 1898. Primer 3W and 3M are, therefore, refered to as the "detection primers," one specific for the wild-type G and the other for the mutant A sequence, respectively. In addition, a mutation of T to G (*underlined*) was introduced at the third position from the 3′ end of the detection primer to further enhance the specificity of the amplification reaction. The sequences for primers 3W and 3M are shown in captal bold letters with the 3′ adjacent sequence in lower case and the core AUG start codon in lowercase boldface.

Serology. HBsAg was measured by Ausria II (Abbott Laboratories, North Chicago, IL); anti-HBc, IgM anti-HBc, HBeAg, anti-HBe, anti-HBs, IgM anti-HAV, and anti-HDV were measured by Corab, Corzyme-M, HBe (recombinant DNA), Ausab, Havab-M and, Anti-delta EIA (Abbott Laboratories), respectively. A second-generation test to detect antibody to hepatitis C virus, either ELISA-2 (Ortho Diagnostic Systems, Raritan, NJ) or anti-HCV peptide EIA (Upstate Biotechnology Incorporated, Lake Success, NY), was performed according to manufacturer instructions. Samples were assayed in duplicate, and only samples positive in duplicate were recorded as reactive. For detection of HCV genomes, RNA was extracted from serum and subjected to reverse transcription PCR amplification using three sets of primers spanning the 5′ noncoding, NS3 and NS5 regions of the HCV genome (12). The PCR products were then subjected to Southern blot hybridization with specific HCV probes (12).

Results

Using oligonucleotide-directed mutagenesis, we have previously generated three mutant HBV genomes with precore mutations: one with the G to A mutation at nt 1901 (GA), the second with the G to A mutation at nt 1898 (AG), and the third with both of the G to A mutations (AA) (13). To demonstrate the utility of ARMDS, we subjected these three HBV mutant DNAs together with the wild-type HBV DNA to the ARMDS analysis. The results are shown in Fig. 2. By either ethidium bromide staining (*A*) or Southern Blot hybridization (*B*), the reactions were specific with respect to the primers used. Using the wild-type primer (primer 3W), we detected amplification signals only in reactions with wild-type and GA mutant DNA (lanes *3, 4, 7,* and *8*), whereas the mutant primer (primer 3M) only amplified AG and AA mutant DNAs (lanes *5, 6, 9,* and *10*).

To assess the potential of the ARMDS method in providing a quantitative measurement of the ratio of the precore mutant to wild-type virus, we mixed wild-type and mutant (AG) HBV DNAs in various proportions and subjected the mixtures to PCR with either primer 3W or 3M and primer 4. The resulting PCR products were analyzed by Southern blot hybridization, and the signals generated by the PCR reaction were subjected to densitometric measurements (Fig. 3). The resulting OD readings from the actual autoradiogram (*A*) were plotted against either the wild-type or mutant DNA concentrations (*B*). The results indicated that the signals generated with either of the primers were fairly linear over a two-log concentration range with respect to the ratios of wild-type vs mutant DNA concentrations. Therefore, using these two primers, we were able to obtain a semiquantitative measurement of the level of the precore mutant with the G to A mutation at nt 1898.

After establishing the sensitivity and specificity of the ARMDS method, we proceeded to analyze some of the serum samples we had characterized previously (Fig. 4). By DNA sequencing, we have previously described the association of the G to A mutation at nt 1898 with an epidemic of fulminant hepatitis B in Israel (7). Serum HBV DNA from five of the

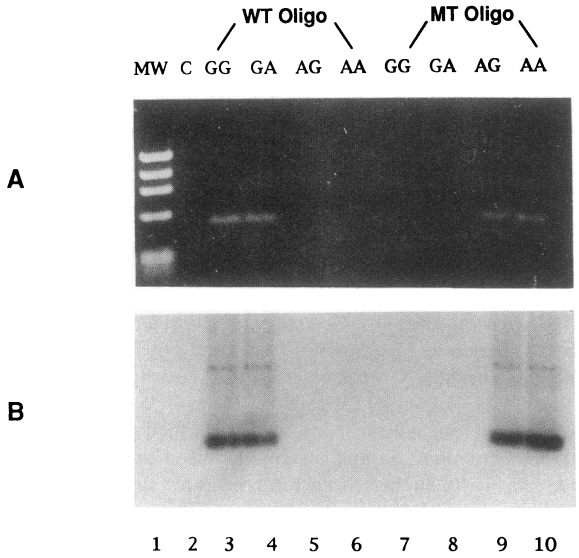

Figure 2. Specific detection of precore mutation by ARMDS. Three mutants HBV DNA together with the wild-type HBV DNA were subjected to the ARMDS analysis. 1 fg of HBV DNA was used in each PCR reaction. The reaction products are electrophoresed, stained with ethidium bromide (*A*), and subjected to Southern blot hybridization with radioactive-labeled HBV DNA probe (*B*). Lane *1* shows molecular weight markers, and lane *2* has no added HBV DNA. Lanes *3* and *7*, wild-type HBV DNA (GG representing nucleotide G at nt positions 1898 and 1901); lanes *4* and *8*, GA mutant DNA; lanes *5* and *9*, AG mutant DNA; and lanes *6* and *10*, AA mutant DNA. Lanes *2–6* represent the product from the PCR reaction using primer 3W (wild-type) and 4, and lanes *7–10* represent the products from primers 3M (mutant) and 4.

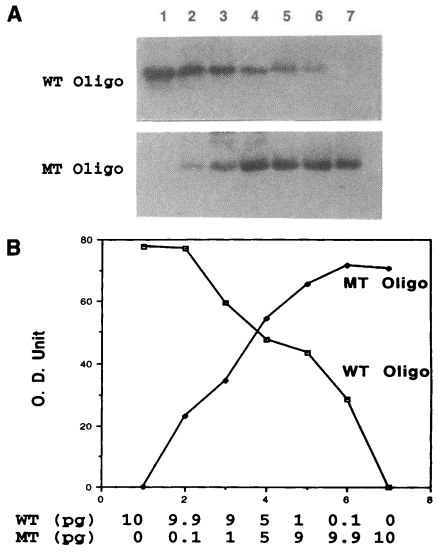

Figure 3. Quantitative analysis of HBV mutant using ARMDS. The wild-type and AG mutant HBV DNAs were mixed in various ratios but kept at a constant total DNA concentration. The mixtures were subjected to ARMDS. The resulting PCR products were analyzed by Southern blot hybridization and subjected to densitometric measurement of the signals generated by the PCR reaction. The resulting OD readings were plotted against the HBV DNA concentrations (*B*). *A* is the actual autoradiogram. Top section of *A* represents reactions with the wild-type detection primer (primer 3W) and the bottom section with the mutant primer (primer 3M). Lanes *1–7* represented PCR reactions using HBV DNA concentrations listed below *B*.

patients were analyzed for the presence of this precore mutation by ARMDS. The index patient, who was the suspected source of the epidemic, and three other patients in whom fulminant hepatitis developed were shown to harbor the mutant genome exclusively (lane *4–7*, respectively), whereas the asymptomatic patient who had minimal acute hepatitis in that report had only the wild-type sequence (lane *8*). These results were in perfect agreement with our previous DNA sequencing data. Seven patients with sporadic fulminant hepatitis described in Table I (patients 1–6 and 24) were analyzed as well, and the results are shown in Figure 4 (lanes *9–15*). In a parallel experiment, we isolated and sequenced four to five independent PCR clones of the precore/core region from each of the patients. The results of DNA sequencing demonstrated that patients 1–6 had the wild-type sequence, and that patient 7 had the mutant sequence at nt position 1898 in all clones. The results of the ARMDS analysis were comparable to the sequencing data, except in patients 2 and 4 (lanes *11* and *13*, respectively), whose clones contained small amounts of the mutant strains. The proportions of mutant to wild-type DNA in either of the two samples were < 1:100, as evidenced by extrapolating the ratio of OD readings on the graph in Fig. 3 *B*. Because only four to five clones from each sample were sequenced, we were not surprised that only the wild-type sequence was detected.

Table II illustrates the results of the ARMDS analysis on all the patients with either fulminant hepatitis B or acute, self-limited hepatitis B. Using the first set of generic HBV primers, we successfully amplified HBV DNA from serum in 28 out of 40 patients with fulminant hepatitis and 16 of 16 patients with acute, self-limited hepatitis in the first PCR reaction. These 28 samples were then subjected to the second round of PCR amplification with sequence-specific primers. A mixture of wild-type and mutant sequences in various ratios was observed in 15 patients with fulminant hepatitis, wild type exclusively in 11, and mutant exclusively in 2. In patients with acute, self-limited

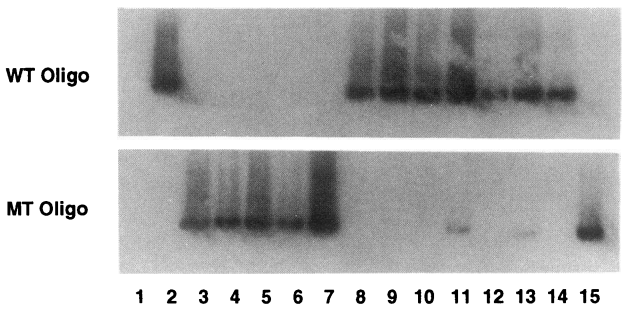

Figure 4. ARMDS Analysis of precore mutation in patients with fulminant hepatitis B. Serum HBV DNA from patients with fulminant hepatitis B were subjected to ARMDS analysis for the presence of the precore mutation as described in the text. Lane *4* is the index patient who was the suspected source of the epidemic, and lanes *5–7* represent three of the patients who developed fulminant hepatitis B in the epidemic. The asymptomatic patient who had minimal acute hepatitis in our paper is represented in lane *8*. Lanes *9–15* represent PCR reactions of patients 1, 24, and 2–6 with sporadic fulminant hepatitis B described in Table I, respectively. Top section represents reactions with the wild-type detection primer (primer 3W) and the bottom section with the mutant primer (primer 3M).

Table I. Clinical Characteristics of Patients with Fulminant Hepatitis B

Patient	Age/Race/Sex	Region	AST/ALT	T bili	PT	Risk factors	OLT	Outcome
			U/liter		*s*			
1	39/W/F	Wash	3361/3086	18.7	ND	IVDA	−	Died
2	42/Hisp/M	Colorado	858/292	26.8	ND	None	−	Died
3	71/W/M	Alabama	2952/ND	19.2	ND	Transfusion	−	Died
4	21/B/F	Florida	4490/6260	32.8	ND	None	−	Alive
5	70/Hisp/M	Colorado	212/1551	10.5	ND	None	−	Died
6	38/W/F	Florida	1587/1708	23.3	ND	None	−	Alive
7	50/W/M	Alabama	211/ND	26.0	ND	None	−	Died
8	22/W/F	N. Engl	4984/ND	19.0	47.4	None	−	Died
9	56/W/M	N. Engl	2700/ND	32.6	46.3	None	−	Died
10	56/W/F	N. Engl	1650/ND	35.0	25.8	None	−	Alive
11	38/W/M	N. Engl	2324/2754	10.8	23.8	None	−	Died
12	58/W/F	M. Atlant	340/367	27.4	19.2	None	−	Died
13	35/Hisp/M	M. Atlant	1320/2354	26.5	43.6	None	−	Died
14	25/B/M	M. Atlant	3964/4349	28.1	28.2	None	−	Died
15	42/W/F	M. Atlant	133/283	9.8	13.4	Sexual	+	Alive
16	64/I/F	Alaska	5790/12100	10.5	56.8	None	−	Died
17	16/W/M	Texas	NA/NA	NA	NA	None	+	Alive
18	23/H/M	Texas	NA/NA	25	28	None	−	Died
19	25/B/F	Texas	1500/600	36	39	IVDA	−	Died
20	53/W/M	M. Atlant	244/123	11.6	>40	None	+	Died
21	52/A/M	M. Atlant	922/589	27.9	21.9	None	+	Died
22	33/A/M	M. Atlant	90/305	15.8	11.9	None	+	Died
23	44/A/M	M. Atlant	73/145	1.7	13.0	None	+	Died
24	37/W/M	Wash	1927/1237	33.0	ND	IVDA	−	Died
25	44/Hisp/M	N. Engl	1975/2367	17.9	29.8	Sexual	+	Alive
26	23/W/F	M. Atlant	1564/ND	24.3	26.5	Sexual	−	Alive
27	38/W/M	M. Atlant	2970/1730	25.2	16.5	None	+	Alive
28	28/W/F	M. Atlant	1305/2515	12.7	>40	IVDA	−	Alive

W, white; A, Asian; B, Black; I, Indian; OLT, orthotopic liver transplantation; AST, aspartate aminotransferase (normal range = 5–40 U/liter); ALT, alanine aminotransferase (normal range = 7–56 U/liter); PT, prothrombin time; T bili, total bilirubin (normal range = 0.3–1.0 mg/dl). The laboratory values shown here represent the serum peak liver function abnormalities during the hospital admission. Patients 1–23 were infected with HBV only, patient 24 had evidence of HAV and HBV coinfection, patients 25 and 26 were coinfected with HBV and HCV, and patients 27 and 28 were coinfected with HBV and HDV.

hepatitis, nine had exclusively wild-type HBV strain and seven had mixtures of WT and mutant in various ratios (Table II). Failure to amplify HBV DNA in 12 serum samples could have several explanations. First, since some of the samples that were quite old (> 15 yr in storage) had been frozen and thawed several times, HBV virion particles could have been disrupted during the process. Second, the primers that we used span a large region of HBV genome (828 bp), which is more difficult to amplify as compared with a smaller region. These two factors could have contributed to a lower sensitivity of detection. Third, the level of HBV viremia is often low during the symptomatic phase of fulminant hepatitis (14), and our sensitivity of detection using PCR is consistent with the findings of other reports (15, 16). Nevertheless, the clinical and serological features of those 12 patients whose serum samples we could not detect HBV DNA were not different from those of patients detailed in Table I. Four of these patients (patients 20–23) had undergone orthotopic liver transplantation for HBV-associated cirrhosis, and fulminant HBV hepatitis developed after transplantation. Pre- and posttransplant serum samples from patient 20 were analyzed: a mixture of wild-type and mutant HBV strains was detected in both samples. Patient 24 had coexisting IgM anti-HAV, suggesting coinfection with HAV and HBV. Patients 25 and 26 had coexisting HCV RNA and anti-HCV, respectively. Anti-HDV was detected in patients 27 and 28.

Discussion

Studies in other laboratories as well as our own have linked the occurrence of fulminant hepatitis to infection by HBV mutants unable to synthesize HBeAg (7–9). In addition, other reports have suggested that this HBV mutant can lead to to a more severe chronic hepatitis and resistance to interferon therapy (17). Some of these associations, however, could be coincidental, reflecting merely the prevalence of this mutant form of HBV infecting those populations under study (18, 19). In this study, we first established that the amplification refractory mutation detection system is a rapid, sensitive, and specific method to detect the G to A mutation at nt position 1898. It also provides a quantitative measurement for the relative ratio of one strain to the other in a sample. The ability to assess quantitatively the level of this specific mutation will be useful in analyzing the fluctuation of circulating amounts of each

Table II. Serologic Markers and HBV Strains in Patients with Fulminant Hepatitis B

Patient	Anti-HAV IgM	Anti-Delta	Anti-HCV	HCV RNA	*HBV Wild type	*HBV Mutant
Fulminant hepatitis						
1	–	–	–	–	10	0
2	–	–	–	–	10	<0.1
3	–	–	–	–	10	0
4	–	–	–	–	10	<0.1
5	–	–	–	–	10	0
6	–	–	–	–	0	10
7	–	–	–	–	10	0
8	–	–	–	–	0	10
9	–	–	–	–	5	10
10	–	–	ND	–	4	10
11	–	–	–	–	10	3
12	–	–	–	–	10	0
13	–	–	–	–	10	0
14	–	–	–	–	10	0
15	–	–	–	–	10	9
16	–	–	–	–	2	10
17	–	–	ND	–	10	0
18	–	–	ND	–	10	0
19	–	–	ND	–	10	0
20	–	–	–	–	10	4
21	–	–	–	–	5	10
22	–	–	–	–	4	10
23	–	–	–	–	2	10
24	+	–	–	–	10	0
25	–	–	ND	+	10	2
26	–	–	+	–	2	10
27	–	+	–	–	5	10
28	–	+	–	–	1	10
Acute, self-limited hepatitis						
1	–	–	ND	ND	10	1
2	–	–	ND	ND	10	0
3	–	–	ND	ND	10	2
4	–	–	ND	ND	1	1
5	–	–	–	ND	10	1
6	–	–	–	ND	10	0
7	–	–	–	ND	1	10
8	–	–	–	ND	1	10
9	–	–	–	ND	10	0
10	–	–	ND	ND	1	1
11	–	–	ND	ND	10	0
12	–	–	ND	ND	10	0
13	–	–	ND	ND	10	0
14	–	–	ND	ND	10	0
15	–	–	ND	ND	10	0
16	–	–	ND	ND	10	0

ND, not done. * Amplification signal intensities were quantitated by densitometer (see Methods) and ratios of OD readings between wild-type and mutant amplification signals were obtained. The higher OD reading of the two in each sample was standardized to a value of 10, and the other value was approximated to the first decimal point based on the ratio of the two actual OD readings.

strain in response to antiviral therapy. In addition, this methodology can be applied easily to the detection of other mutations in the viral genome. Similarly, sequence-specific oligonucleotides have been used to determine semiquantitatively the relative prevalence of each viral strain in a mixture of viral strains (19). Our technique, however, has the advantage of revealing the presence of a small percentage (< 5%) of a viral strain in the mixture.

Using this ARMDS method, we detected both wild-type and mutant HBV strains in patients with sporadic fulminant hepatitis in the United States. In addition, no appreciable difference in the clinical course of disease was observed between those patients with and without mutant infection. The argument can be made that since we were detecting only the G to A mutation at nt 1898, we could have missed other mutations affecting the precore open reading frame and resulting in the absence of HBeAg production. Other such mutations have been described (5, 6); however, we have independently cloned and sequenced the precore region in eight cases of our study cohort and noted wild-type sequences in seven of them. Therefore, we conclude that the wild-type HBV strain, at least with respect to HBeAg synthesis, is often detected in patients with fulminant hepatitis B. Our observation is also supported by other studies (20, 21). Furthermore, both wild-type and mutant HBV strains could be detected in patients with acute, self-limited hepatitis B. However, the majority of patients harbored predominantly wild-type strain and no patients had exclusively mutant form. Since serum samples from many of our cases were collected and stored in the distant past, it was not possible for us to obtain well-matched controls for our study cases in each geographic area. Nonetheless, our study demonstrated unambiguously that both wild-type and mutant HBV strains are associated with fulminant hepatitis, and that other host or viral factors are important in the development of fulminant hepatitis in the United States. However, in this study, we cannot exclude a possible contribution of the HBV precore mutant to the development of more severe liver disease.

Four of the patients (patients 20–23) underwent orthotopic liver transplantation for HBV-associated cirrhosis, and fulminant hepatitis developed after transplantation. In these patients, reinfection by the same HBV strain(s) probably resulted in the rapid course of hepatic failure. A mixture of HBV strains was detected in their serum samples after transplantation. One patient, patient 20, had serum samples taken before and after transplantation; analysis of these two samples revealed a mixture of both strains in approximately equal ratios. These findings suggest that the mutant strain did not have a selection advantage over the wild-type strain, and that the rapid hepatic deterioration of liver disease that occurred after transplantation was not a direct result of allograft reinfection by the mutant virus. These conclusions, however, were derived from observations in immunosuppressed transplantation recipients; therefore, we cannot necessarily extrapolate our conclusions to immunocompetent patients with acute hepatitis B.

One of our patients had evidence of acute coinfection by hepatitis A virus and two by hepatitis D virus. Patient 24 coinfected with HAV was an intravenous drug addict (IVDA) and had wild-type virus exclusively in his serum. A mixture of wild-type and mutant strains was detected in both of the HDV coinfected patients (patients 27 and 28); one (patient 28) of the two had a history of parenteral exposure as an IVDA. Two patients (patients 25 and 26) had serologic and/or PCR evidence of coinfection with HCV, and both of them had potential sexual exposure as the only identifiable risk factor. Con-

trary to results in a previous study from Europe, in which 8 of 17 patients with fulminant hepatitis B had evidence of HCV infection (16), we did not find such a high prevalence of HCV markers in American patients with acute fulminant hepatitis B. The presence of both HBV and HCV markers in two of our patients probably reflects the well-known epidemiological association of both viral infections (22, 23). Since there are no serologic tests yet available to distinguish between acute and chronic HCV infection, whether acute HCV infection contributed to the development of fulminant hepatitis in these patients remains to be established.

The pathophysiologic and clinical significance of the G to A precore mutation is still largely unknown at present. Conceivably, this mutation may alter the regulation of HBV transcription and replication by providing a more thermodynamically stable packaging signal for the encapsidation of HBV pregenomic RNA (24, 25). Data from our laboratory and others (13, 26), however, suggest that these mutations do not affect viral protein synthesis, transcription, or replication in transfected human hepatoma cells. Woodchuck hepatitis virus variants containing this precore mutation appeared to replicate normally in woodchucks (27). Instead, the absence of HBeAg production as an immune target may confer a biological advantage to the virus as it encounters the immune response in infected host; indeed this may be the major reason for the emergence of these HBV precore mutants. Within the precore open reading frame, there are several possible codons that can be mutated to a stop codon resulting in the abrogation of HBeAg synthesis (26). Why the mutation occurs predominantly in the codon involving nt 1898 is not apparent at present. This can be explained potentially by preferential mutagenesis targeting to this nucleotide during HBV replication. Since the precore region also contains the Direct Repeat-1 and the encapsidation signal, mutations resulting in other potential stop codons may exert deleterious effect on the replication of HBV; if they are lethal mutations, with no evolutionary advantage, they would not become clinically detectable. A similar G to A mutation has been observed in the viral genome during replication of hepatitis D virus both in tissue culture and in infected hosts (28). It is tempting to speculate about the existence of a putative "virulence factor" associated with certain strains of HBV; such a viral property could potentially affect the clinical course of infection and possibly constitute the molecular basis for the diverse manifestations of HBV-induced liver disease.

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