

Expression of Defective Hepatitis B Virus Particles Derived from Singly Spliced RNA Is Related to Liver Disease

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Background. Defective hepatitis B virus (HBV) particles, generated from singly spliced HBV RNA, have been detected in chronic carriers of HBV. The present study was designed to quantify the expression of defective HBV (dHBV) and wild-type HBV (wtHBV) genomes in the serum of patients with HBV infection and its relation to the severity of liver disease.

Methods. HBV and dHBV loads were determined by quantitative polymerase chain reaction in the serum of 89 untreated HBV-infected patients (31 coinfecting with human immunodeficiency virus [HIV] type 1) with liver disease of different stages. The ratio of dHBV DNA to total (wtHBV plus dHBV) HBV DNA (dHBV/HBV ratio) was used to express data independently of the level of viral replication.

Results. Despite a global correlation between dHBV and wtHBV load, the dHBV/HBV ratio ranged from 0.001% to 69%. The variation in dHBV/HBV ratio was independent of HIV coinfection, HBV genotype, and precore mutations. The mean dHBV/HBV ratio was higher in patients with severe liver necrosis and fibrosis.

Conclusions. Our data indicate that an elevated dHBV/HBV ratio is associated with liver necroinflammation and fibrosis disease, suggesting a regulation of dHBV expression according to the severity of the liver disease. The dHBV/HBV ratio may help to better define liver disease stage during HBV infection.

Persistent human hepatitis B virus (HBV) infection is a major public health problem. The major complications of chronic HBV infection are the development of liver cirrhosis and hepatocellular carcinoma (HCC) [1–3]. Evidence indicating a direct involvement of HBV in this process is now available [3]; indeed, recent data have demonstrated that serum HBV DNA levels are strong predictors of the risk of HCC, independent of hepatitis B e antigen (HBeAg) expression, serum alanine amino-

transferase (ALT) level, and liver cirrhosis [4]. Moreover, retrospective studies have shown that HBV genotype and HBeAg expression may also increase the risk of HCC [5, 6]. In addition, the effect of HBV genetic variability, including precore (preC) mutants and viral genotype, has been implicated in liver disease progression [7]. Furthermore, HIV-1 infection in HBV-infected patients increases the level of HBV DNA and the risk of cirrhosis [8]. HBV should therefore be considered as having synergistic effects with chronic inflammation in the pathogenesis of liver disease.

HBV is a small, enveloped DNA virus and is a member of the hepadnavirus family; its genome consists of a relaxed, circular, partially double-stranded 3.2-kb DNA molecule [9]. Five unspliced messenger (mRNA) transcripts of 3.5 (short and long), 2.4, 2.1, and 0.8 kb are synthesized from the HBV genome and encode for the capsid, envelope, polymerase, and transactivator X viral proteins [9]. The 3.5-kb short pregenomic mRNA is also involved in the HBV replication cycle via its reverse tran-

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Table 1. Clinicopathological data on patients with chronic hepatitis B virus (HBV) infection (n = 89).

Parameter	HBV mono-infection	HBV/HIV coinfection
Sex, no. male/female	43/15	29/2
Age, years	34 ± 10 (19–81)	36 ± 5 (22–50)
HIV load, copies/mL	...	8.8 × 10 ⁴ ± 5.1 × 10 ⁴ (<500–323,500)
CD4 cell count, cells/mm ³	...	428 ± 250 (45–856)
Alanine aminotransferase level, IU/L	107 ± 15 (17–610)	74 ± 14 (16–374)
METAVIR histological score, no. of patients		
Liver necroinflammation		
A0–A1	28	6
A2–A3	30	13
Liver fibrosis		
F0–F2	27	21
F3–F4	31	10

NOTE. Data are means ± SEs (ranges), unless otherwise specified.

scription, which occurs in core particles [9]. In addition to unspliced HBV mRNA, singly and doubly spliced 2.2-kb mRNAs arising from 3.5-kb pregenomic mRNA have been identified in HBV DNA-transfected cell lines and in HBV-infected liver [10–16]. The major spliced HBV mRNA that lacks intron 2447/489 accounts for up to 30% of pregenomic transcripts, as revealed in transfected cells and in the livers of chronically infected patients [10–13]. The mechanism for the regulation of splicing is still not clearly understood. Unlike the retrovirus model, the influence of HBV protein expression and viral genetic variability on splicing regulation has never been investigated. However, a recent report suggested that the posttranscriptional regulatory element, which is involved in the nuclear export of intronless viral subgenomic RNAs, presents regulatory elements that control viral pregenome processing by stimulating or inhibiting splicing [17]. We and others have shown that spliced mRNA is packaged, reverse transcribed, and leads to the secretion of defective viral particles [14, 15, 18–20]. Defective viruses are maintained in the population through transcomplementation with wild-type helper virus [18]. The regulation of spliced HBV RNA packaging is not well defined; however, recent data have indicated that mutations within the C-terminus of HBV core protein may influence the encapsidation of spliced HBV RNA [21, 22]. In addition, the processing of defective HBV (dHBV) secretion may differ from that of wild-type HBV (wtHBV) secretion [20].

Defective HBV genomes contain open reading frames that may encode for various fusion and truncated proteins. It has been reported that a polymerase and surface fusion protein encoded by spliced RNA is incorporated into viral particles and may then play a role in the viral life cycle [16]. In addition, it has also been demonstrated that the expression of spliced duck HBV RNA encoding for the large surface protein is important for replication in infected hepatocytes [23]. In woodchucks chronically infected with woodchuck hepatitis virus (WHV), 2 different

spliced WHV RNAs potentially encoding for core and polymerase fusion proteins were secreted as defective particles [24, 25].

The most frequently detected dHBV genomes result from the reverse transcription of singly spliced 2.2-kb mRNAs [14]. We previously demonstrated that this spliced HBV genome leads to the *in vivo* expression of a new HBV splice-generated protein (HBSP), which corresponds to fusion of the N-terminal part of polymerase and a new open reading frame created by the single splicing event [26]. HBSP expression did not modulate the level of HBV replication, but indirect *in vivo* detection of HBSP may be related to viral load and liver fibrosis [27].

Overall, it is clear that dHBV particles are present in the liver and serum of chronically infected patients. However, the clinical impact of their expression during liver disease remains to be established. The aim of the present study was, therefore, to quantify dHBV and wtHBV particles in serum samples and to evaluate the impact of HBV genetic variability and liver status on dHBV particle expression in untreated individuals with chronic HBV infection.

METHODS

Patients and samples. Eighty-nine chronic HBV carriers who were positive for hepatitis B surface antigen, had a detectable HBV load, were negative for markers of hepatitis C and D virus, and had never received anti-HBV therapy were randomly included in our study (table 1). Untreated patients were selected to avoid any bias resulting from HBV fitness due to antiviral treatment. Of the selected patients, 31 were coinfecting with HIV-1. HIV coinfection is usually associated with rapid progression to liver cirrhosis [8]. ALT activity and HBeAg levels were evaluated by standard biological assays (bioMérieux). For HBV/HIV-coinfecting patients, CD4 cell count (Coulter) and HIV load (Bayer) were quantified. Liver biopsy specimens were obtained during a 3-month period around the date of serum sample, em-

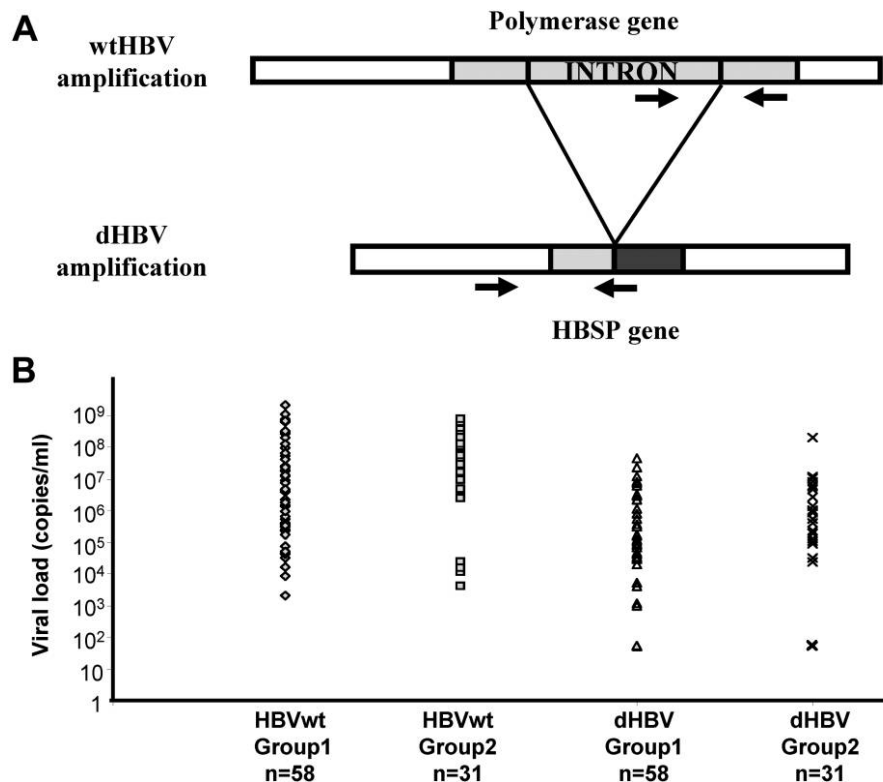


Figure 1. A, Schematic representation of wild-type hepatitis B virus (wtHBV) DNA and defective HBV (dHBV) DNA generated from singly spliced 2.2-kb HBV pregenomic RNA. For wtHBV, the gene encoding for polymerase is shown in light gray; for dHBV, the HBV splice-generated protein (HBSP) gene, in frame with polymerase, is shown in light gray, whereas the 3' part encoding for the original sequence is shown in dark gray. Arrows indicate the location of primers for specific amplification of either wtHBV or dHBV DNA. B, wtHBV and dHBV loads in patient groups 1 (HBV mono-infection) and 2 (HBV/HIV coinfection). Values for wtHBV and dHBV loads were determined by real-time quantitative polymerase chain reaction and are represented using logarithmic scales.

bedded in paraffin, and sectioned; sections were stained with hematoxylin-eosin. Liver biopsy specimens were classified as exhibiting no or moderate (A0–A1) or severe (A2–A3) liver necrosis and no or moderate (F0–F2) or severe (F3–F4) liver fibrosis, using the METAVIR scoring method. For 12 patients with HIV-1 coinfection, the fibrosis score was evaluated by high-resolution real-time ultrasound on the basis of a quantitative scoring system. Necroinflammation scores were not available for these 12 patients.

Real-time polymerase chain reaction (PCR). HBV DNA was extracted from serum samples (200 μ L) by means of a spin column (Qiagen) and eluted in 200 μ L of water. Real-time quantitative PCR was performed using the LightCycler system (Roche) in 20 μ L of LightCycler DNA Master SYBR Green Mix (Roche) containing 3 mmol/L MgCl₂ and 2 μ L of viral DNA extract. The dHBV primers were 5'-AGTGTGGATTTCGCACTCC-3' (forward; nt 2271–2289) and 5'-CTGGTTGTTGATGATCATT-3' (reverse; nt 504–489[^]2447–2445); the wtHBV primers, forward 5'-ATCTTCTTGTTGGTTCCTTCT-3' (forward; nt 430–450) and 5'-CTGAAGCCAAACAGTGG-3' (reverse; nt 733–715). Each PCR cycle comprised denaturation at 95°C for 20 s, annealing at 65°C for 15 s, and extension at 72°C for 12 s (45 cycles). The standard

curve for quantification was calculated using serial 10-fold (500 to 10¹¹ copies/mL) dilutions of wtHBV or dHBV plasmids.

Analysis of HBV genotypes and preC mutants. To detect mutations in the HBV basal core promoter (BCP) and preC regions and to determine HBV genotypes, a 644-bp fragment including the entire BCP (nt 1742–1849) and preC (nt 1814–1900) regions was amplified using the primers 5'-CATGGACCACCGTGAAC-3' (forward; nt 1605–1621) and 5'-AGTGCGAATCCACACTC-3' (reverse; nt 2284–2268) and was sequenced in an Applied Biosystems 3100 automatic sequencer. In the BCP region, mutations in nt 1762–1764 were analyzed; in the preC region, all nucleotide changes were analyzed. Viral genotypes were established by aligning the preC/core (preC/C) sequences obtained with consensus sequences of HBV genotypes A, B, C, D, E, F, and G from GenBank.

Statistical analysis. Statistical analysis was performed using the nonparametric Mann-Whitney *U* test; results are given as means \pm SEs and medians. The correlation assay was evaluated by the Spearman rank correlation test (version 5.0; StatView software) (SAS). Analysis of variance was performed to compare HBV genotype. Differences were considered significant at *P* < .05. The Bonferroni correction was used to determine the *P* value cutoff for liver status (ALT activity, liver necroinflammation, and fibrosis).

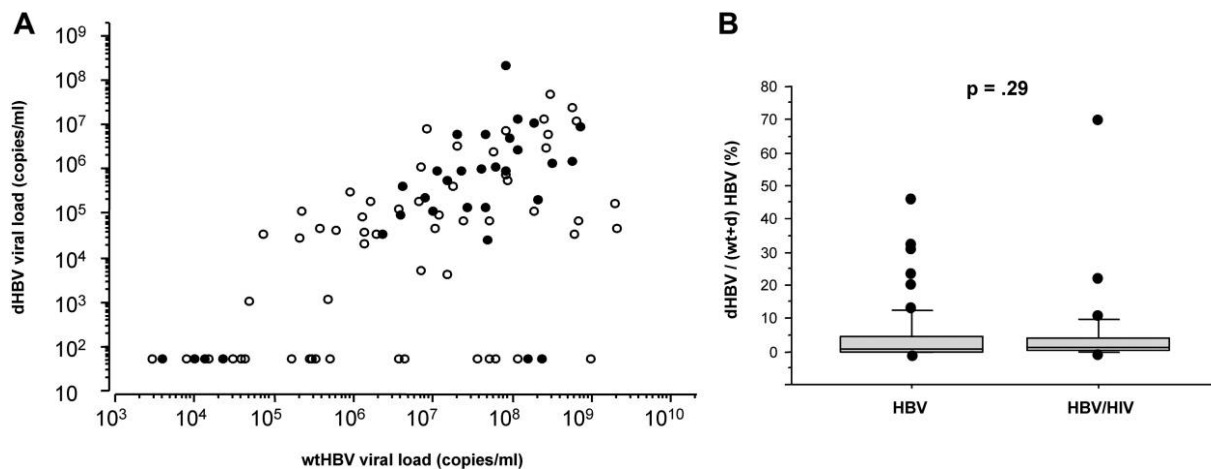


Figure 2. A, Relationship between the level of defective hepatitis B virus (dHBV) DNA and that of wild-type HBV (wtHBV) DNA in serum samples from 89 chronic HBV carriers. Black circles correspond to HBV/HIV-coinfected patients (group 2), and white circles correspond to HBV-monoinfected patients (group 1). Values for wtHBV and dHBV loads were determined by real-time quantitative polymerase chain reaction and are represented using logarithmic scales. When dHBV DNA was undetectable, an arbitrary value of 50 copies/mL (10-fold below the threshold of detection) was attributed. B, Box plot of the distribution of the dHBV/HBV ratios (i.e., the ratio of dHBV DNA to total [wtHBV plus dHBV {wt+d}] HBV DNA) in groups 1 and 2. Ratios are expressed as percentages.

RESULTS

Quantification of dHBV and wtHBV DNA in the serum of chronic HBV carriers. dHBV and wtHBV DNA were quantified by real-time PCR in the serum samples of patients either infected with HBV alone (group 1; $n = 58$) or coinfecting with HBV and HIV-1 (group 2; $n = 31$) (table 1). To quantify wtHBV DNA specifically, one of the primers was located inside the intronic sequence, whereas, for dHBV DNA amplification, one of the primers encompassed splice donor and acceptor sites (figure 1A). The set of primers for dHBV was unable to amplify wtHBV DNA plasmid, thus demonstrating the specificity of these primers (data not shown). All samples tested were positive for wtHBV DNA, with mean \pm SE viral loads of $1.7 \times 10^8 \pm 0.5 \times 10^8$ and $1.1 \times 10^8 \pm 0.3 \times 10^8$ copies/mL in groups 1 and 2, respectively (figure 1B). The level of dHBV DNA was significantly lower than that of wtHBV DNA in both groups. Defective viral DNA was detected in 73% of samples (viral load ≥ 500 copies/mL), with means \pm SEs of $3.1 \times 10^6 \pm 1.3 \times 10^6$ and $10 \times 10^6 \pm 7.9 \times 10^6$ copies/mL in groups 1 and 2, respectively (figure 1B).

Both dHBV and wtHBV DNA are generated from reverse transcription of HBV pregenomic mRNA, either unspliced or singly spliced [10–16]. Thus, as expected, a correlation was observed between the levels of dHBV and wtHBV DNA expression for all patients ($r = 0.57$; $P < .0001$) and in groups 1 ($r = 0.55$; $P < .0001$) and 2 ($r = 0.54$; $P = .003$) (figure 2A). Strikingly, despite this correlation, defective particles were detected in 2 of 12 patients with low replication (wtHBV load $< 1 \times 10^5$ copies/mL). In addition, in 14 of 77 patients with high replication (wtHBV load $> 1 \times 10^5$ copies/mL), no dHBV particles were detected. These data suggest that dHBV expression is

related to more than just the level of HBV replication and raise the question of the relationship between the regulation of HBV splicing and viral liver disease.

Ratio of dHBV to total HBV particles in the serum of chronic HBV carriers. To study dHBV expression independent of viral replication, the ratio of dHBV DNA to total (wtHBV plus dHBV) HBV DNA (dHBV/HBV ratio) was compared for each patient. To take into account patients with undetectable dHBV DNA, an arbitrary value of 50 copies/mL (10-fold below the threshold of detection) was attributed. The dHBV/HBV ratios were similar in both patient groups (for group 1, mean \pm SE of $4.8\% \pm 1.2\%$, range of 0.001%–46% and median of 0.8%; for group 2, mean \pm SE of $5.1\% \pm 2.3\%$, range of 0.001%–69%, and median of 1.3%; $P = .29$) (figure 2B). The absence of a difference between the groups suggested that HIV-1 infection has no impact on HBV splicing events and the formation of defective particles. Thus, both HBV and HBV/HIV groups were pooled to explore whether the variations observed in dHBV/HBV ratios (from 0.001% to 69%) might be related to HBV genetic status or liver disease.

The dHBV/HBV ratio and HBV status. HBV genotype and mutations in the preC/C region have been implicated in the modulation of viral replication and HBeAg expression [7, 28, 29]. Thus, we wanted to investigate whether viral genotype, mutations in the preC/C region, and the level of HBeAg expression were involved in the variation in the dHBV/HBV ratio. In our cohort, 86% of the HBV samples belonged to HBV genotype A, C, or E (figure 3A). Statistical analysis did not demonstrate a difference when the dHBV/HBV ratio and HBV genotypes were analyzed (figure 3A). For the preC G1896A stop codon and the BCP A1762T and G1764A mutations, no significant difference

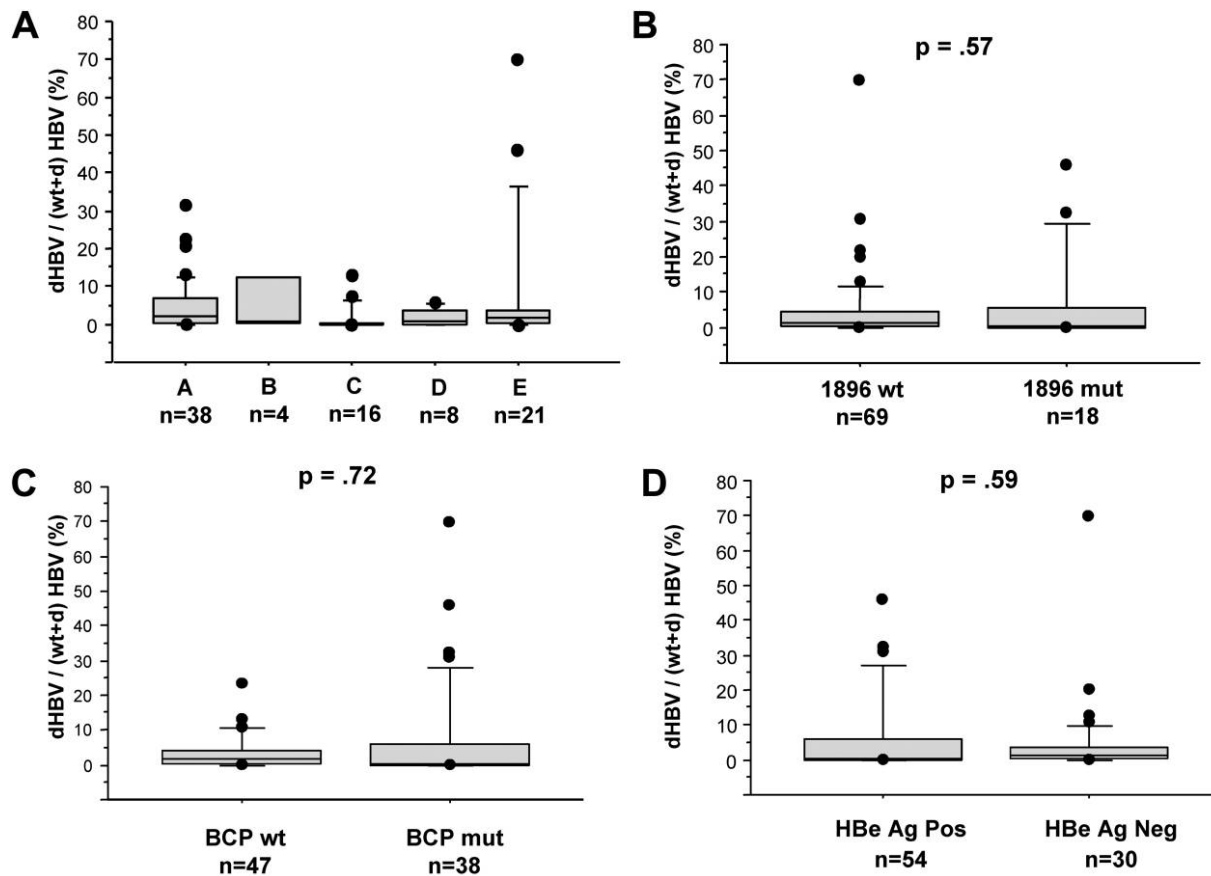


Figure 3. dHBV/HBV ratios (i.e., the ratio of defective hepatitis B virus [dHBV] DNA to total [wild-type HBV plus dHBV {wt+d}] HBV DNA) according to viral status. Ratios are expressed as percentages. Panel A shows a box plot of dHBV/HBV ratios according to HBV genotype. HBV genotypes (A, B, C, D, and E) were determined for 87 of 89 chronic HBV carriers on the basis of sequence analysis of the capsid gene. In our cohort, 86% of HBV samples belonged to HBV genotypes A, C, or E. No significant difference was found between genotypes A, C, and E by analysis of variance. For HBV genotypes B and D, recruitment was insufficient to allow for statistical analysis. Box plots are also shown for dHBV/HBV ratios as functions of the G1896A mutation (mut) in the precore/core sequence (B), of the basal core promoter (BCP) A1762T and G1764A mutations (C), and of hepatitis B e antigen (HBeAg) expression (D). Statistical analysis was performed with the nonparametric Mann-Whitney *U* test. Neg, negative; Pos, positive.

was observed when the dHBV/HBV ratios were compared (figure 3B and 3C). Finally, the dHBV/HBV ratio was not significantly different between HBeAg-positive (mean \pm SE, 4.0% \pm 1.3%; median, 1.3%) and HBeAg-negative (mean \pm SE, 6.2% \pm 2.1%; median, 0.5%) patients ($P = .59$) (figure 3D). These data suggest that the dHBV/HBV ratio is not influenced by mutations usually involved in the modulation of HBV replication.

The dHBV/HBV ratio and liver disease. The next step was to study the relationship between HBV-associated liver diseases and the dHBV/HBV ratio. No significant difference could be seen in the dHBV/HBV ratio according to ALT activity (for ALT level less than twice the normal value, mean \pm SE of 3.2% \pm 0.7% and median of 0.7%; for ALT level more than or equal to twice the normal value, mean \pm SE of 5.7% \pm 2.4% and median of 1.4%; $P = .71$) (figure 4A). However, dHBV/HBV ratios were significantly higher in patients with severe fibrosis (mean \pm SE, 8.1% \pm 2.3%; median, 1.8%) than in those with moderate fi-

brosis (mean \pm SE, 2.2% \pm 0.5%; median, 0.9%) ($P = .04$) (figure 4B). Furthermore, dHBV/HBV ratios were significantly higher in the population with severe liver necrosis (mean \pm SE, 7.6% \pm 2.1%; median, 1.8%) than in those with moderate or no liver necrosis (mean \pm SE, 1.8% \pm 0.6%; median, 0.3%) ($P = .009$) (figure 4C). The 4-fold increase in mean dHBV/ HBV ratio in patients with severe liver necrosis was a consequence of a 2-fold decrease in wtHBV load and a 2-fold increase in dHBV load. However, no significant difference was observed between patients with moderate or no liver necrosis and those with severe liver necrosis for either wtHBV load (mean \pm SE, $2.4 \times 10^8 \pm 0.9 \times 10^8$ and $0.9 \times 10^8 \pm 0.3 \times 10^8$ copies/mL, respectively) or dHBV load (mean \pm SE, $2.5 \times 10^6 \pm 1.5 \times 10^6$ and $6.2 \times 10^6 \pm 4.6 \times 10^6$ copies/mL) ($P = .64$). Similar results were observed for fibrosis (data not shown). Thus, our data support the notion of a specific regulation of dHBV expression according to the severity of liver disease.

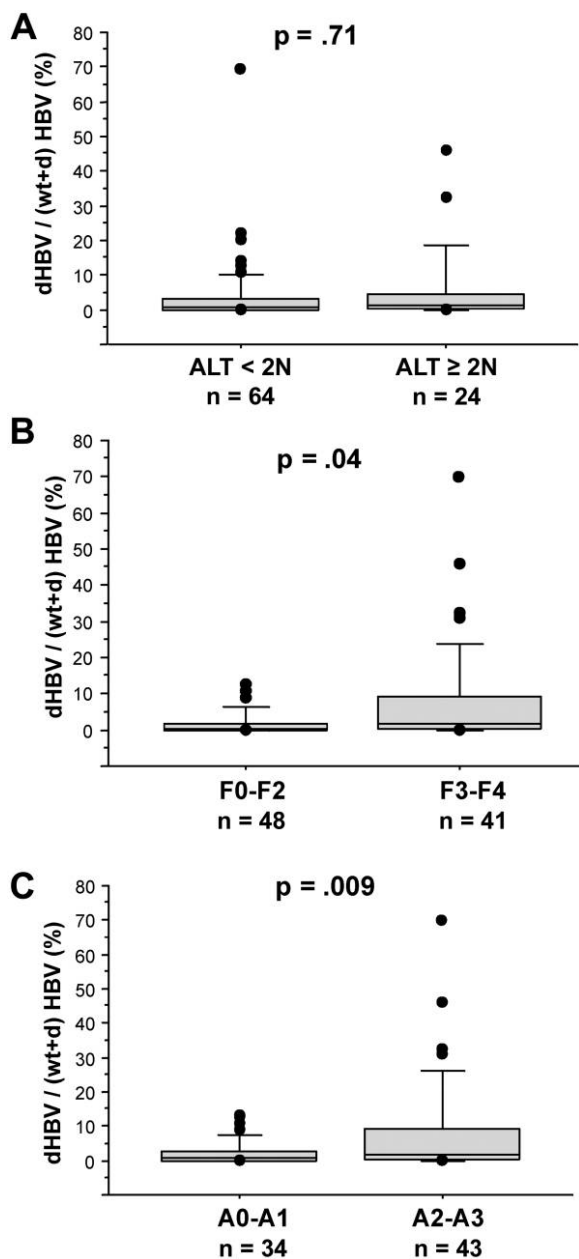


Figure 4. dHBV/HBV ratios (i.e., the ratio of defective hepatitis B virus [dHBV] DNA to total [wild-type HBV plus dHBV {wt+d}] HBV DNA) according to liver disease status. Ratios are expressed as percentages. Shown are box plots of dHBV/HBV ratios as a function of alanine aminotransferase (ALT) activity (*A*), of liver fibrosis status (*B*), and of liver necroinflammatory histological status (*C*). ALT activity was considered positive when the ALT level was at least twice the normal value (2N). Liver biopsy specimens were classified as having no or moderate (A0–A1) or severe (A2–A3) liver necrosis and no or moderate (F0–F2) or severe (F3–F4) liver fibrosis, using the METAVIR scoring system. Statistical analysis was performed with the nonparametric Mann-Whitney *U* test.

DISCUSSION

The present study was performed to investigate the regulation of dHBV particle expression in HBV-related liver disease. Indeed, although a study using nonquantitative methods showed a de-

crease in the ratio of spliced to unspliced RNA in patients with high versus low histologic activity indexes [30], regulation of dHBV particle expression remained unclear. Thus, levels of wtHBV and dHBV genomes were quantified in serum samples from patients with chronic HBV infection with or without HIV-1 coinfection. In patients with active HBV replication (wtHBV $>1 \times 10^5$ copies/mL; 86% of studied patients), the dHBV load was $>1 \times 10^5$ copies/mL in 56% of cases. This result raises the question of the function of splicing events and the formation of defective particles in the natural history of HBV infection.

The dHBV/HBV ratio is a measure independent of the level of viral replication. The percentage of dHBV particles varied from 0.001% to 69% of the total viral circulating population. Clearly, for most patients, the circulating amount of dHBV particles was low in comparison with wtHBV particles. The mean dHBV/HBV ratio was far from what was expected, considering the reported intracellular proportion of spliced pregenomic RNA ($\sim 30\%$ of HBV pregenomic mRNA) [10–13]. This difference might be explained, as reported elsewhere, by the intracellular retention of defective particles compared with wild-type virus secretion [20]. Altogether, despite a correlation between dHBV and wtHBV particles, dHBV expression might depend on more than the HBV replication level. The dHBV/HBV ratio was not influenced by HIV-1 coinfection, HBV genotype, or mutations in the basal core or preC regions. In contrast, we found that the dHBV/HBV ratio was significantly elevated in serum samples obtained from patients with severe liver fibrosis or necrosis. However, the difference in the dHBV/HBV ratio regarding fibrosis is at the limit of statistical significance ($P = .04$) and is not significant after the Bonferroni correction (threshold of $P = .016$). This might reflect the inclusion of HIV-coinfected patients in our cohort. Indeed, it has been reported previously that HIV infection influenced the progression of liver fibrosis without increasing liver necroinflammation in HBV chronic carriers [8]. When HIV-coinfected patients were excluded from the analysis, the relationship between the dHBV/HBV ratio and liver fibrosis was more notable (median, 1.8% vs. 0.1%; $P = .02$ for the difference, which is the limit of significance using the Bonferroni correction). Nevertheless, these data suggest a direct association between the dHBV/HBV ratio and the severity of liver disease.

A lack of correlation between liver histology and single measurements of HBV DNA level has been reported previously [31]. However, effective suppression of viral replication in patients with chronic HBV infection has been shown to slow disease progression and improve patient outcomes [32]. Furthermore, loss of viral control in patients infected with HBV strains resistant to antiviral treatment leads to more rapid disease progression [33]. These data illustrate that serum HBV DNA level is a predictive factor for cirrhosis and HCC. In addition, a recent report showed that, in untreated chronic HBV carriers, the risk of cir-

rhosis increased with increasing HBV DNA level, independent of serum ALT level or HBeAg status [34].

Taken together, our data reveal a correlation between dHBV expression and HBV replication capacity but also indicate a specific regulation of dHBV expression that is related to the severity of liver disease. In this context, reports have highlighted the modulation of splicing events in cellular gene expression during the progression of liver disease [35, 36]. In addition, it has recently been suggested that HBV RNA splicing could be modulated by the splicing factor PSF [17]. Consequently, it could be hypothesized that the expression of defective particles is modulated by the extracellular environment of hepatocytes during liver disease.

During the course of HBV infection, the mechanisms of liver injury are usually attributed to the host immune response. However, evidence now suggests that expression of certain HBV proteins has a direct impact on liver disease [3]. Our data show that the regulation of dHBV particles is linked to the severity of viral liver disease. These defective particles may be involved in liver disease through either the modulation of viral protein expression or the expression of spliced specific viral protein. Indeed, we have shown previously that singly spliced HBV RNA and its related defective particles allowed overexpression of HBV core antigen and generated the expression of HBSP [26, 37]. We have also demonstrated previously that HBSP is involved in the modulation of cell viability [26]. Further studies are required to determine the direct involvement of HBSP in the pathogenesis of liver disease.

The monitoring of HBV-related liver disease remains a major challenge in clinical practice. Recent reports have demonstrated that liver necroinflammation and fibrosis can be identified by noninvasive tests using different combinations of blood markers [38, 39]. The present study highlights the possibility that the dHBV/HBV ratio might be modulated during liver disease. However, the dHBV/HBV ratio must be investigated in a follow-up study to evaluate the modulation of this ratio during the progression of liver disease. Finally, a prospective clinical study will make it possible to evaluate whether this ratio can be considered, along with biological and virological parameters, to help define the clinical stages of liver disease during HBV infection.

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