Hepatitis B Virus Pregenomic RNA Is Present in Virions in Plasma and Is Associated With a Response to Pegylated Interferon Alfa-2a and Nucleos(t)ide Analogues

L. Jansen, Neeltje A. Kootstra, Karel A. van Dort, R. Bart Takkenberg, Hendrik W. Reesink, and Hans L. Zaaijer

Background. Treatment of patients with chronic hepatitis B (CHB) with nucleos(t)ide analogues (NAs) suppresses hepatitis B virus (HBV) DNA production but does not affect the synthesis of the RNA pregenome or HBV messenger RNA. Whether HBV RNA-containing particles continue to be secreted into the bloodstream remains controversial.

Methods. We developed a sensitive polymerase chain reaction (PCR) assay to quantify the HBV RNA load in a supernatant of NA-treated HepG2-2.2.15 cells and in plasma specimens from 20 patients with CHB who were receiving NA therapy and 86 patients treated with pegylated interferon alfa (Peg-IFN) and adefovir.

Results. Treatment of HepG2-2.2.15 cells with NAs for 9 days reduced HBV DNA levels (by 1.98 log_{10} copies/mL), whereas HBV RNA levels remained higher than HBV DNA levels. Peg-IFN–based treatment induced a stronger decrease in the HBV RNA load than NA monotherapy, and this decline was more pronounced in responders than in nonresponders. In HBV e antigen–negative patients, a lower baseline plasma HBV RNA level was independently associated with response to Peg-IFN and adefovir (odds ratio, 0.44; P = .019). Immunoprecipitation with HBV core antigen–specific antibodies after removal of the HBV surface antigen envelope demonstrated the association of plasma HBV RNA with virions.

Conclusions. HBV RNA is present in virions in plasma specimens from patients with CHB. HBV RNA levels vary significantly from those of established viral markers during antiviral treatment, which highlights its potential as an independent marker in the evaluation of patients with CHB.

Keywords. chronic hepatitis B; HBV life cycle; nucleocapsids; immunoprecipitation; response marker.

Hepatitis B virus (HBV) infection causes a wide spectrum of clinical manifestations, ranging from fulminant acute hepatitis to chronic infection with varying degrees of liver disease [1]. In clinical practice, one can currently choose between 2 types of available therapies for patients with chronic hepatitis B (CHB): pegylated interferon alfa (Peg-IFN) or nucleos(t)ide analogues (NAs) [2, 3].

Peg-IFN exhibits both immunomodulatory and direct antiviral effects, resulting in sustained suppression of HBV DNA after treatment in a subgroup of patients [3]. In contrast, the use of NAs potently reduces the HBV DNA level and its associated complications in most patients [4]. However, blocking reverse transcription by NAs does not affect the formation of HBV pregenomic RNA (pgRNA) or production of viral proteins, such as HBV surface antigen (HBsAg), as the HBV covalently closed circular DNA (cccDNA) is unaffected and remains transcriptionally active [5]. Consequently, treatment discontinuation of NAs usually results in recurrence of disease activity and most patients will probably need lifelong therapy [6].

Whether HBV pgRNA–containing nucleocapsids continue to be enveloped and secreted despite strong inhibition of HBV DNA synthesis during NA therapy remains controversial. It is generally believed that the presence of HBV DNA in nucleocapsids is required to trigger modifications in the capsid structure that allow envelopment and secretion. This is mainly based on in vitro studies that could not detect single-stranded RNA-containing particles in supernatant of human hepatoma cells transfected with an HBV mutant lacking polymerase activity, which eliminates the ability to synthesize HBV DNA [7, 8]. This was further supported by cryo–electron microscopy findings of significant differences in the structure of RNA- and DNA-containing nucleocapsids [9].

In contrast with the belief that pgRNA-containing nucleocapsids are excluded from virion formation, detection of HBV RNA in plasma during therapy with NAs has been reported [10–13]. However, large heterogeneity in methods and findings exists between these studies, which may have accounted for the
lack of acknowledgement of the presence of HBV RNA in peripheral blood. Furthermore, the kind of particles in which HBV RNA would exist in plasma is unclear.

Here, we studied the presence and nature of HBV RNA in plasma by developing a polymerase chain reaction (PCR)–based assay to specifically quantify HBV RNA. First, proof of concept was provided in vitro by evaluating an HBV-producing cell line treated with NAs. This method was then used to study HBV RNA kinetics in plasma specimens from patients with CHB who were receiving long-term NAs or treatment with Peg-IFN and adefovir. Furthermore, characterization of secreted HBV RNA–containing particles was provided by immunoprecipitation with antibodies to HBsAg or HBV nucleocapsid (HBcAg).

**PATIENTS AND METHODS**

**Subjects**

**Patients With CHB Receiving Long-term NA Therapy**

We selected 20 patients with CHB from the database of the Laboratory of Clinical Virology (Academic Medical Center) who had started long-term NA monotherapy (Table 1). In total, 10 HBV e antigen (HBeAg)–positive patients (5 were receiving entecavir, and 5 were receiving tenofovir) and 10 HBeAg-negative patients (5 were receiving entecavir, and 5 were receiving tenofovir) who had started long-term NA treatment were included.

Plasma samples collected before the initiation of NA therapy (mean time of collection [±SD], −3.9 ± 4.1 weeks) and approximately 30 weeks (mean time [±SD], 28.0 ± 7.2 weeks), 60 weeks (mean time [±SD], 62.6 ± 11.9 weeks), and 120 weeks (mean time [±SD], 123.6 ± 16.3 weeks) after the start of therapy. In addition, plasma samples from 3 HBeAg-positive patients with CHB with high HBV DNA levels (>8.00 log_{10} copies/mL) were used for validation of the HBV RNA assay. All samples were stored at −80°C until assayed.

**Patients With CHB Treated With Peg-IFN and Adefovir Combination Therapy**

In total, 92 patients with CHB and HBV DNA levels of >100 000 copies/mL (17 182 IU/mL) participated in a prospective investigator-initiated study. Detailed study characteristics have been described elsewhere [14]. In summary, patients were treated for 48 weeks with 180 µg of pegylated interferon alfa-2a subcutaneously once weekly and 10 mg of adefovir dipivoxil daily. After 48 weeks, treatment was discontinued, and a treatment-free follow-up period was started. The study was conducted according to the guidelines of the Declaration of Helsinki and the principles of good clinical practice and was approved by local ethics committees (ISRCTN 77073364). All patients gave written informed consent.

Inclusion criteria for the present study were completion of 48 weeks of treatment and 2 years of follow-up. Of the 92 patients treated in the initial study, 86 fulfilled these criteria (Table 1 and Supplementary Figure 1). In addition, 23 of these patients were selected for longitudinal analysis of HBV RNA levels during treatment (13 were positive for HBeAg, and 10 were negative for HBeAg).

Achievement of a combined response and/or HBsAg loss (HBsAg level of < 0.05 IU/mL) was determined after 24 weeks (week 72) and 2 years (week 144) of treatment-free follow-up.

**Table 1. Baseline Characteristics of Patients With Chronic Hepatitis B in the 2 Studied Cohorts, by Treatment Group and Hepatitis B Virus e Antigen Status**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Long-term NAa</th>
<th>Peg-IFN and Adefovir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBeAg Positive (n = 10)</td>
<td>HBeAg Negative (n = 10)</td>
</tr>
<tr>
<td>Demographic</td>
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<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>39.6 ± 13.6</td>
<td>53.1 ± 6.2</td>
</tr>
<tr>
<td>Female sex</td>
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<tr>
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<td>5 (50)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>NA naive</td>
<td>5 (50)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT level, × ULN</td>
<td>3.7 ± 3.9</td>
<td>3.3 ± 2.8</td>
</tr>
<tr>
<td>HBV DNA load, log_{10} copies/mL</td>
<td>8.97 ± 0.84</td>
<td>6.89 ± 1.07</td>
</tr>
<tr>
<td>HBsAg, log_{10} IU/mL</td>
<td>4.24 ± 0.64</td>
<td>3.46 ± 0.48</td>
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<tr>
<td>HBV genotype</td>
<td></td>
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</tr>
<tr>
<td>A</td>
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<td>2 (20)</td>
</tr>
<tr>
<td>B</td>
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<tr>
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<td>3 (30)</td>
</tr>
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</table>

Data are mean value ± SD or no. (%) of patients.

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide analogue; Peg-IFN, pegylated interferon; ULN, upper limit of normal.

a Five patients in each group were receiving entecavir, and 5 in each group were receiving tenofovir.
Combined response was defined as HBeAg loss in HBeAg-positive patients and as HBV DNA levels of ≤2000 IU/mL and persistent normal alanine aminotransferase levels in both HBeAg-positive and HBeAg-negative patients [3].

**Cell and Culture Conditions**
The HBV inhibitory activity of tenofovir (Gilead Sciences) and entecavir (Bristol-Myers Squibb) was analyzed in the hepatoma cell line HepG2 2.2.15, which is stably transfected with the HBV genome and produces Dane particles, as well as HBeAg, HBsAg, and HBV core particles [15, 16].

In brief, HepG2 2.2.15 cells were grown in William’s E medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamin, and 5 μM dexamethasone and were maintained at 37°C in a 5% CO2 humidified atmosphere. Cells were seeded in 6-well plates at a density of 0.3 × 10⁶ cells/mL. Two days after reaching confluency, entecavir (1 mM) or tenofovir (10 mM) in William’s E medium was added (day 0). For all culture conditions, medium was changed at days 0, 3, and 6, and fresh medium, with or without drug, was added. Total HBV DNA and HBV RNA levels were measured in supernatant at days 0, 3, 6, and 9 of the experiment.

**Quantification of HBV RNA**
For the detection of HBV RNA in plasma, we modified a transcript-specific quantitative PCR (qPCR) assay previously described [17]. A detailed protocol is described in the Supplementary Materials.

Briefly, total RNA was isolated from plasma or culture supernatant, using the QIAamp Viral RNA Mini Kit (Qiagen), and treated with DNase (Promega). RNA was concentrated using the NucleoSpin RNA Clean-up XS kit (Macherey-Nagel) and reverse transcribed with M-MLV reverse transcriptase (Promega) with an HBV-specific primer. The determination of HBV RNA levels was performed by qPCR in a LightCycler2000 system (Roche), using HBV RNA–specific primers (designed to detect both pgRNA and precore [PC] messenger RNA [mRNA]) and SYBR Green as a reporter dye. Complementary DNA was quantified by comparing the signals to a standard curve. To ensure that no HBV DNA was measured, qPCR was performed in parallel on RNA samples without reverse transcription (hereafter referred to as “no-RT controls”). The lower limit of quantification (LoQ) was determined at 357 copies/mL (2.55 log₁₀ copies/mL), and the lower limit of detection (LoD) was determined at 71 copies/mL (1.85 log₁₀ copies/mL), when using 280 µL of plasma or supernatant as input.

**Other Virological Analyses**
Quantitative HBsAg levels were determined by the Architect assay (Abbott, Abbott Park, Illinois), with an LoD of <0.05 IU/mL. Quantitation of plasma HBV DNA levels was done using the Cobas TaqMan 48 assay (Roche), with a dynamic range between 20 and 1.70 × 10⁶ IU/mL. Signals of <20 IU/mL were set at 10 IU/mL (58 copies/mL or 1.76 log₁₀ copies/mL) for statistical analysis. The presence of PC or basal core promoter (BCP) mutations was determined by sequencing the BCP/PC region with dideoxynucleotide technology.

**Immunoprecipitation of HBV Particles**
Immunoprecipitation of viral particles was performed using μMACS technology (Miltenyi Biotec) according to the manufacturer’s protocol, with the following modifications. Cell debris was removed from 140 µL of HepG2 2.2.15 supernatant or patient plasma by high-speed centrifugation (20,800 × g at 4°C for 5 minutes). Samples were subsequently precleared by incubation with 50 µL of Protein G MicroBeads at room temperature for 2 hours. After application on a μMACS column, the flow-through pool was incubated with 2 µg of a monoclonal antibody against HBsAg (Abnova; catalog no. MAB5402) on ice for 30 minutes and subsequently with 50 µL of Protein G MicroBeads on ice for another 30 minutes.

Alternatively, samples were treated with 1% nonionic detergent Nonidet P-40 (NP40) and 10 mM dithiothreitol (DTT) on ice for 30 minutes to remove the viral envelope and were then incubated with 2 µg of a monoclonal antibody against HBCAg (Abnova; catalog no. MAB5399). Following application on a new μMACS column and washing with phosphate-buffered saline, bead-bound fractions were eluted from the columns by applying lysis buffer for viral DNA or RNA isolation.

**Statistical Analysis**
Differences between groups were examined using the Student t test, and correlations of parameters were determined by Spearman rank correlation. Multivariable logistic regression was used to assess HBV RNA as independent predictor of therapy response. P values of <.05 were considered statistically significant. Statistical comparisons were performed using IBM SPSS Statistics, version 19.0.0.1 (SPSS, Chicago, Illinois).

**RESULTS**

**HBV RNA Assay Validation in Untreated Chronic Hepatitis B Patients**
We validated our HBV RNA assay in plasma samples obtained at a single time point from 3 treatment-naïve HBeAg-positive patients with CHB who had a high HBV DNA load (Figure 1). HBV RNA was present in high concentrations in plasma (range, 4.85–6.10 log₁₀ copies/mL). Treatment of plasma with RNAse before isolation did not affect HBV RNA levels, which excludes the presence of free HBV RNA. Omitting reverse transcription to control for residual DNA after DNAse treatment (no-RT controls) resulted in HBV RNA levels that were <0.1% of the original value. In addition, PC mRNA levels were <1% of the total pgRNA and PC mRNA levels. Both findings indicate that the large majority of HBV RNA measured is pgRNA. Furthermore, HBV RNA quantification was reproducible, showing comparable values after 3 independent isolations of the same sample (Supplementary Figure 4).
HBV DNA and HBV RNA Levels in NA-Treated HepG2 2.2.15 Cells

Incubation of HepG2 2.2.15 cells with different concentrations of entecavir or tenofovir for 9 days resulted in a marked reduction in the HBV DNA level in supernatant, compared with untreated cells, after correction for cell count at each time point (Supplementary Figure 5). No significant differences were observed between cells treated with entecavir or tenofovir, which were combined for further analysis. The HBV DNA level in supernatant of cells treated with entecavir (1 mM) or tenofovir (10 mM) was reduced by up to 1.98 log_{10} copies/mL, compared with untreated cells (P < .001; Figure 2A). In contrast, HBV RNA levels increased by 0.47 log_{10} copies/mL at day 9 in supernatant of NA-treated cells (P < .05; Figure 2B).

Plasma HBV RNA Levels During Treatment With NAs or Peg-IFN and Adefovir Combination Therapy

HBV RNA levels at baseline and during treatment were determined in a cohort of NA-treated patients (n = 20) and in a subset of 13 HBeAg-positive patients (7 with a combined response and 6 with no response) and 10 HBeAg-negative patients (5 with a combined response and 5 with no response) treated with Peg-IFN and adefovir.

HBV RNA Levels in NA-Treated HBeAg-Positive Patients

HBV RNA was detectable in all HBeAg-positive patients before treatment (mean ± standard error of the mean, 6.1 ± 1.2 log_{10} copies/mL). HBV RNA levels declined less than HBV DNA levels, and mean HBV RNA levels were significantly higher than HBV DNA levels during all time points during treatment (Figure 3A). At the end of follow-up (week 120), 7 of 10 patients had HBV DNA levels below the LoD, whereas only 1 of 10 patients had HBV RNA levels below the LoD (mean ± standard error of the mean, 2.0 ± 0.1 vs 3.4 ± 0.4 log_{10} copies/mL, respectively; P = .002).

In total, 4 of 10 patients became HBeAg negative during NA treatment (after 58, 189, 195, and 214 weeks). Patients who became HBeAg negative had significantly lower HBV RNA levels than those who remained HBeAg positive at all time points during treatment (Supplementary Figure 7).

**Figure 1.** Plasma hepatitis B virus (HBV) RNA levels in 3 untreated patients with chronic hepatitis B. HBV RNA loads were measured in 3 different conditions: in untreated plasma, after RNAse treatment of plasma, or without reverse transcription (RT) of RNA. In addition, precore (PC) messenger RNA (mRNA) was quantified. Bars represent mean ± standard error of the mean. *P < .05 and **P < .001 by paired t tests. Abbreviation: pgRNA, pregenomic RNA.

**Figure 2.** Hepatitis B virus (HBV) DNA and RNA levels in nucleos(t)ide analogue (NA)–treated HepG2 2.2.15 cells. A, HBV DNA levels in culture supernatant of NA-treated and untreated HepG2 2.2.15 cells. Data represent mean ± standard error of the mean of 3 replicates in each condition (untreated, entecavir 1 mM, and tenofovir 10 mM). B, HBV RNA levels in the same culture supernatant. Values represent the difference between NA-treated and untreated cells in log_{10} copies/mL at each time point. *P < .05 and **P < .001.
HBV RNA Levels in Peg-IFN and Adefovir-Treated HBeAg-Positive Patients

Similar to the kinetics in NA-treated patients, in HBeAg-positive patients treated with Peg-IFN and adefovir, the decline of plasma HBV RNA levels was less pronounced than that of HBV DNA levels (Figure 3B). In addition, HBeAg-positive patients with a combined response at week 72 showed a stronger decline in plasma HBV RNA levels than nonresponders. Mean HBV RNA levels of responders were lower than those of nonresponders at all time points during treatment, a difference that was significant from week 30 onward.

HBeAg-positive patients treated with Peg-IFN and adefovir showed a stronger decline in HBV RNA levels than patients receiving NA monotherapy after 30 weeks of treatment (Figure 3C). In contrast, the decline in HBV DNA levels was not significantly different (Figure 3D).

HBV RNA Levels in NA-Treated HBeAg-Negative Patients

Plasma HBV RNA was present in all HBeAg-negative patients before treatment (Figure 4A). The decrease of HBV DNA levels was stronger than that of HBV RNA levels (mean decline [±SEM] at week 30, 4.4 ± 0.4 vs 1.0 ± 0.2 log10 copies/mL, respectively; P < .001). All HBeAg-negative patients had undetectable HBV DNA levels at the end of follow-up (week 120). However, as opposed to HBeAg-positive patients, the majority of HBeAg-negative patients (8 of 10 at week 20) had HBV RNA levels below the limit of detection, as well.

HBV RNA Levels in Peg-IFN and Adefovir-Treated HBeAg-Negative Patients

HBeAg-negative patients with a combined response had lower HBV RNA levels than nonresponders before and during treatment with Peg-IFN and adefovir (Figure 4B).
In addition, responders showed a stronger and earlier decline in HBV RNA. At week 6 of treatment, all responders (5 of 5) had HBV RNA levels below the LoQ, whereas HBV RNA levels remained above the LoQ in most (4 of 5) nonresponders (mean [±SEM], 1.8 ± 0.2 vs 3.7 ± 0.7 log10 copies/mL, respectively; \( P = .028 \)).

Baseline HBV RNA Levels and Response to Therapy
In the cohort of 86 patients with CHB treated with Peg-IFN and adefovir, HBV RNA levels strongly correlated with HBV DNA levels in both HBeAg-positive (n = 41) and HBeAg-negative (n = 45) patients before the start of treatment. In contrast, HBV RNA levels only showed a moderate correlation with HBsAg levels in HBeAg-positive patients, and no correlation was observed in HBeAg-negative patients (data not shown).

In HBeAg-negative patients, baseline HBV RNA levels were lower in patients who achieved a combined response at week 72, as compared to nonresponders (mean [±SEM], 3.68 ± 0.29 vs 4.60 ± 0.20 log10 copies/mL; \( P = .01 \)). HBeAg-negative patients with a combined response at week 72 also had significantly lower HBsAg and HBV DNA levels and were more often (peg-)interferon experienced. In multivariable analysis, baseline HBV RNA load was a predictor of response, independent of HBsAg level or past (peg-)interferon exposure (Table 2).

In HBeAg-positive patients, baseline HBV RNA levels were not associated with a combined response at week 72, as compared to nonresponders (mean [±SEM], 3.68 ± 0.29 vs 4.60 ± 0.20 log10 copies/mL; \( P = .01 \)). HBeAg-positive patients with a combined response at week 72 also had significantly lower HBsAg and HBV DNA levels and were more often (peg-)interferon experienced. In multivariable analysis, baseline HBV RNA load was a predictor of response, independent of HBsAg level or past (peg-)interferon exposure (Table 2).

Immunoprecipitation Analysis of the Association of HBV RNA With Viral Particles
To investigate what kind of particles harbor HBV RNA, we performed immunoprecipitation experiments, using HepG2 2.2.15 supernatant or patient plasma. We used antibodies to HBsAg...
(anti-HBs) to specifically isolate HBV virions or subviral particles and antibodies to HBCAg (anti-HBc) to isolate nucleocapsids.

**Immunoprecipitation of HepG2 2.2.15 Supernatant**

First, precipitation of supernatant from both treated and untreated HepG2 2.2.15 cells with anti-HBs was performed. Treatment with NAs did not affect HBV RNA levels, which remained detectable at high concentrations in the anti-HBs precipitated fraction of the culture supernatant (Figure 5A). In contrast, HBV DNA levels after anti-HBs precipitation declined significantly (by $2.07 \log_{10}$ copies/mL; $P < .001$). Anti-HBs–associated HBV RNA levels were significantly higher than HBV DNA levels in NA-treated cell supernatant (5.34 vs 3.59 $\log_{10}$ copies/mL, respectively; $P < .001$), indicating that the majority of produced virions during NA treatment contained HBV RNA and not HBV DNA.

**Immunoprecipitation of Plasma Specimens From Patients With CHB**

We performed a similar experiment with plasma specimens from 3 untreated patients with CHB and a high viral load. Unfortunately, precipitation with anti-HBs was not successful in plasma specimens from these patients. After precipitation with anti-HBs, HBV DNA (as a positive control) was detectable in very low levels only (data not shown), suggesting inefficient precipitation of HBV virions with anti-HBs by using this method.

As an alternative, we pretreated samples with a detergent (NP40-DTT) to remove the viral envelope and release the capsids, allowing subsequent precipitation of capsids with anti-HBc. Indeed, NP40-DTT treatment significantly enhanced the detectability of HBV DNA and RNA. Only a small fraction (<10%) of HBV RNA remained in the flow-through pool and could not be precipitated with anti-HBc (Figure 5B), indicating that the majority of HBV RNA measured was associated with nucleocapsids. In addition, precipitated HBV RNA levels increased by approximately 100-fold after NP40-DTT treatment, compared with untreated plasma, suggesting that the large majority (>99%) of HBV RNA–containing capsids were enveloped.

**DISCUSSION**

In this study, we showed that HBV RNA is present in plasma specimens from patients chronically infected with HBV and remains present in higher levels than HBV DNA during NA therapy. In contrast, patients receiving Peg-IFN and adefovir combination therapy showed a stronger decline in HBV RNA level than NA-treated patients, and the extent of the decrease in HBV RNA level was associated with response to therapy. Importantly, immunoprecipitation with anti-HBs and anti-HBc indicated that the majority of HBV RNA is present in HBV virions.

Achievement of viral clearance (ie, HBsAg loss) rarely occurs in the treatment of patients with CHB with currently available treatment modalities, and reactivation of disease activity is common after treatment cessation [2, 3]. Thus, additional virological markers are needed that reflect the proportion or activity of intrahepatic cccDNA, to better monitor or predict treatment response.

In this study, we set up a method modified from Laras et al [17] to quantify HBV RNA in plasma specimens from patients with CHB and found that plasma HBV RNA represents a plasma marker distinct from other commonly used virological markers. We were able to detect HBV RNA in plasma specimens from nearly all (105 of 106) untreated patients with CHB included
in this study, with levels that were strongly correlated with HBV DNA levels in untreated patients, comparable to previous reports [10, 18].

During NA therapy, HBV DNA levels showed a much stronger decline than HBV RNA levels, resulting in mean HBV RNA levels that were significantly higher than HBV DNA levels during all time points during treatment. This was most striking in HBeAg-positive patients who remained HBeAg positive during longer follow-up. In accordance with recent reports [18, 19], we observed a stronger HBV RNA load decline in HBeAg-positive patients who became HBeAg negative during NA therapy. Furthermore, patients who were HBeAg negative before NA therapy showed a strong decline in HBV RNA levels during NA therapy, as well.

In line with these observations was the stronger decline in HBV RNA levels in HBeAg-positive patients with a combined response (including HBeAg loss) after treatment with Peg-IFN and adefovir, compared with nonresponders. However, differences in HBV RNA were significant only from 30 weeks of treatment onward. In HBeAg-negative patients, lower plasma HBV RNA levels were associated with a combined response already before the start of therapy. This effect was independent of baseline HBsAg and previous IFN treatment in multivariable analysis, which highlights a possible role for HBV RNA levels in predicting response to IFN-based therapy.

We observed that patients receiving Peg-IFN and adefovir combination therapy had a stronger decline in HBV RNA levels than those receiving NA monotherapy. Interestingly, even HBeAg-positive patients without HBeAg loss who were receiving Peg-IFN had stronger a decrease in the HBV RNA load than NA-treated patients. This finding fits with data from previous studies showing that IFN exerts multiple antiviral effects, such as epigenetic cccDNA modifications and prevention of the formation of pgRNA-containing capsids [20].

Despite several reports on HBV RNA in peripheral blood, it remained unclear what kind of particles harbor HBV RNA. One previous study detected HBV RNA in the same fraction as HBcAg and HBV DNA in a sucrose gradient analysis, suggesting the presence of HBV RNA in viral particles [10]. In contrast, in vitro studies concluded that HBV RNA–containing nucleocapsids could not be enveloped and secreted [7, 8, 21]. Our experiments with HepG2 2.2.15 cells, however, clearly showed that both HBV DNA– and HBV RNA–containing particles could be precipitated with anti-HBs, indicating the presence of HBV RNA in virions. Furthermore, a relative increase in virion-related HBV RNA over HBV DNA was observed during NA treatment, indicating that the majority of virions contained HBV RNA during NA treatment. This finding does not necessarily contradict previous in vitro studies because these studies did not include NA-treated conditions and because HBV RNA was only present in a minority of total virions in untreated conditions.

Importantly, we were also able to associate HBV RNA with virions in plasma specimens from patients with CHB by immunoprecipitation. Initially, anti-HBs precipitation was not successful in patient plasma. The fact that it was not successful for both HBV DNA and HBV RNA suggests the presence of inhibiting factors for anti-HBs precipitation, such as HBsAg/anti-HBs immune complexes [22] or the relative excess of subviral (HBsAg-negative) particles in patient plasma [23]. To overcome this issue, we pretreated plasma samples with a detergent to remove the viral envelope and release the capsids. We showed that this treatment significantly increased the HBV RNA yield after subsequent precipitation with anti-HBc, which indicates that HBV RNA in plasma is present in viral particles containing both a capsid and an envelope.

The finding of an excess of HBV RNA over HBV DNA in plasma specimens from NA-treated patients raises questions regarding its consequences. The question remains whether virions harboring an HBV RNA genome can be infectious. Interestingly, it was previously shown that cells transfected with a hepadnaviral RNA pregenome were able to produce complete infectious viruses [24]. If RNA-containing viruses are able to infect hepatocytes, this in theory could lead to the production of new DNA-containing viruses. Efficient infection models and specific isolation of HBV RNA–containing particles would be needed to study this in future experiments.

In conclusion, we showed that HBV RNA is detectable in plasma specimens from both treated and untreated patients with CHB. The influence of NA therapy on plasma HBV RNA levels is limited, despite a strong reduction in HBV DNA levels. In HBeAg-positive patients, decline in HBV RNA levels during therapy predicted NA-induced or Peg-IFN–induced HBeAg loss, whereas HBeAg-negative patients with a combined response to Peg-IFN had lower HBV RNA levels at baseline. The association of HBV RNA with viral particles increases the possibility of a distinct role for plasma HBV RNA in HBV pathogenesis and highlights the need for additional studies on its virological and therapeutic characteristics.

Supplementary Data
Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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H. L. Z. and H. W. R. designed the study. L. J., K. A. v. D., and R. B. T. acquired the data. L. J. and N. A. K. analyzed and interpreted the data and drafted the manuscript. K. A. v. D., R. B. T., H. W. R., and H. L. Z. critically revised the manuscript.

Potential conflicts of interest. H. W. R. received grants from and is a consultant for Roche, Bristol Myers Squibb, and Gilead Sciences. R. B. T. received grants from Roche and Bristol Myers Squibb and is a consultant for Gilead Sciences. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential

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