Naturally occurring core-gene-defective hepatitis B viruses

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This study was undertaken to determine the prevalence of core-gene-defective hepatitis B virus (HBV) in patients with chronic HBV infection, and the nature and significance of the deletions. PCR was performed on sera from 263 patients with chronic HBV infection. Seventeen (6.5%) patients had smaller band(s) in addition to a band of the expected size. Additional bands were also detected in the follow-up samples from 80% and 3% patients who had respectively, multiple and single bands initially. Six patients were further studied by direct sequencing. Four patients had in-frame deletions leading to loss of codons 79-122 of the core gene. Two patients had identical frameshift deletions of nucleotides 2204-2333 resulting in the loss of the first nine codons of the overlapping P gene. Follow-up samples from three of four patients studied showed deletions identical to those in the initial samples. The persistence of these deletions suggests that they were stable and that they may contribute to the chronicity of infection.

Defective virus genomes lack adequate function in one or more of the essential genes required for viral replication (Huang & Baltimore, 1970). They depend on helper activity from another virus gene(s) (cis-rescue) or protein(s) (trans-complementation) for replication. Defective viruses may attenuate the virulence of wild-type virus by interfering with wild-type virus replication or by modulating the immune response thus facilitating the persistence of infection (Huang & Baltimore, 1970; Holland, 1990).

Naturally occurring deletions in hepatitis B virus (HBV) genomes have been reported by several investigators. Most of the deletions are located in the pre-S gene (Gerken et al., 1991; Santantonio et al., 1992; Minami et al., 1993; Nakijima et al., 1994; Melegari et al., 1994). Deletions in the core gene that can generate defective viruses have also been found in patients with chronic HBV infection (Okamoto et al., 1987; Wakita et al., 1991), but the prevalence and clinical significance of these defective genomes remain uncertain. In Okamoto’s study, all four patients were asymptomatic hepatitis B e antigen (HBeAg) positive carriers. However, in Wakita’s study, core gene deletions were detected in patients with chronic hepatitis only. Viruses from the sera of all four patients in Okamoto’s study, but only one of the seven in Wakita’s study were cloned and sequenced. Most of the deletions were located in the centre of the core gene, but detailed characterization of the deletions was not available for all the clones.

This study was undertaken to determine (1) the prevalence of core-gene-defective HBV in a large cohort of patients with chronic HBV infection, (2) the location and nature of the deletions, and (3) the clinical significance of these defective viruses.

All the patients who were studied in a previous report on pre-core HBV mutations were included (Lok et al., 1994). They comprised 263 Chinese patients with chronic HBV infection. At presentation, 201 patients were HBeAg-positive, and 62 were HBeAg-negative. The earliest available sample of residual serum from each patient was used for PCR amplification of HBV DNA. None of the patients had received interferon therapy prior to the collection of the initial serum samples. Follow-up samples from 129 patients, after an interval of at least 1 year, were also analysed.

HBV serological markers were tested by using commercial ELISA kits from Abbott Laboratories. DNA extraction and PCR was performed as described previously (Lok et al., 1994) except for a change in anti-sense primers to include the entire core gene. The sense primers were P1 and P3, and the anti-sense primers were EP2 (5’ GTAGAAGAATAAAGCCCC 3’, positions 2503-2487) and C2 (5’ ATACTAACATTGACATCC- C 3’, positions 2455-2436). To prevent cross-contamination, all precautions recommended by Kwok & Higuchi (1989) were observed and negative controls were included in each assay.

Samples that yielded more than one band on ethidium-
bromide-stained agarose gels of the second round PCR products were further examined if the bands were widely separated. PCR products were loaded on 1% low melting agarose gels. Two bands were cut from each sample: the band of expected size and the fastest migrating band. The agarose slices were melted and the amplified HBV DNA was recovered with a Prep-A-Gene kit (Bio-Rad). After elution, DNA was precipitated with ethanol and then resuspended for direct sequencing.

Each serum sample was amplified in two different nested PCR assays to yield sufficient DNA for sequencing, and to avoid errors introduced by Taq polymerase. Dideoxynucleotide termination sequencing was performed with the Sequenase kit (version 2.0, US Biochemical) using the following primers: sense, P3 (positions 1774–1798), S2 (5'-TTTGCTTCTGACTTCCTTCTTTC 3', positions 1954–1973), S3 (5'-GCACTCAGGCAAAGCTATTC 3', positions 2060–2078) and C1 (5'-GGAAAGAAGTCAGAAGGCAA 3', positions 2267–2289); anti-sense, P5 (5'-GGAAGAAGTCAAGGGCAAA 3', positions 1974–1955), P4 (5'-GGCGAGGGAGTTCTTCTTC 3', positions 2388–2364) and C2 (positions 2455–2436). The entire core gene sequences of the expected and fastest migrating bands for each sample were determined and compared.

Of the samples from the 263 patients studied, 246 (93.5%) had one band and 17 (6.5%) more than one band on ethidium-bromide-stained agarose gels of PCR products from their initial samples (Fig. 1). All 17 patients with multiple bands had a band of the expected size, and additional band/bands that were smaller in size. In all 17 patients, the bands of expected size and the fastest migrating bands were similar in intensity, while the bands of intermediate size were usually fainter. At presentation, 184 (75%) patients with single bands and all 17 with multiple bands were HBeAg-positive (Table 1). There was no difference in sustained loss of HBeAg during follow-up between the patients with single (33%) vs multiple (24%) bands ($P = 0.6$).

Follow-up samples were available in 10 of the 17 patients who had more than one band initially: eight had multiple bands in the follow-up samples that were similar in size to the ones in the initial samples; two had the band of the expected size only in the follow-up samples. Of the eight patients who had persistent multiple bands, four received interferon therapy during follow-up: one had interferon-induced and three had spontaneous sustained loss of HBeAg. Of the two patients who had a band of the expected size only in the follow-up samples, both remained HBeAg-positive; one received interferon therapy during follow-up.

Follow-up samples were available in 119 of the 246 patients who had a single band initially: 116 (97%) had a single band of the expected size, and three (3%) had more than one band in the follow-up samples. All three patients who developed additional band(s) and 71 (61%)...
Table 1. Demographic data, HBe status, serum alanine aminotransferase level (ALT), liver histology, dates of interferon therapy (IFN Rx) and subsequent HBe seroconversion in patients who had multiple bands

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Date</th>
<th>ALT (U/l)</th>
<th>No. of bands</th>
<th>IFN Rx</th>
<th>Loss of HBeAg</th>
<th>Histology*</th>
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<td>1</td>
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<td>32</td>
<td>8/84</td>
<td>+ 40</td>
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<td>-</td>
<td>CAH</td>
</tr>
<tr>
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<td>12/87</td>
<td>+ 60</td>
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<td>-</td>
<td>1/89</td>
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</tr>
<tr>
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<td>M</td>
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<tr>
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<tr>
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<td>F</td>
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<td>&gt; 1</td>
<td>8/87-1/88</td>
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<td>NSRH</td>
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</table>

* NSRH, non-specific reactive hepatitis; CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; NA, not available.

Patients who continued to have a single band had received interferon therapy during follow-up. None of the three patients who developed additional bands during follow-up responded to treatment.

Six patients with widely separated bands were further studied. The fastest migrating band for each patient had a major deletion and a variety of other mutations and minor deletions. The location of the deletions is illustrated in Fig. 2. Patients 3–6 had in-frame deletions of 114, 105, 105 and 87 bp leading to loss of codons 85–122, 79–113, 79–113 and 79–107, respectively. There was significant overlap in the location of the deletions in these four patients. Patient 5 had an additional 36 bp in-frame deletion leading to loss of nucleotides 2027–2062 (codons 43–54). The overlapping P gene was unaffected in these four patients.

Patients 1 and 2 had identical frameshift deletions—nucleotides 2204–2333 (codons 101–144)—which resulted in the loss of the first nine codons of the overlapping P gene. Interestingly, both defective viruses had a potential start codon for the P gene exactly nine codons upstream of the major deletions (Fig. 2). Patient 1 had an additional mutation which changed codon 84 from TTA to TGA thus creating a stop codon for the core gene. Patient 2 had an additional 10 bp frameshift deletion (nucleotides 2152–2161) which created a premature stop codon for the core gene at codon 92.

The sequence of the undeleted parts of the core gene in the fastest migrating bands of these six patients had one to six nucleotide changes and zero to four amino acid changes compared to the consensus sequence; there were zero to four nucleotide changes and zero to three amino...
acid changes compared to the expected size bands from the same patients.

Follow-up samples from four of the six patients from whom viruses were sequenced were also analysed (Fig. 2). The follow-up samples from three patients (1, 2 and 6) had exactly the same deletions as the initial samples. The fastest migrating band of the follow-up sample from patient 5 had one in-frame deletion only that partially overlapped with the major deletion of the initial sample: nucleotides 2163–2306 (cf. nucleotides 2134–2238) (Fig. 2). Although the two samples were collected 73 months apart, this patient had no change in HBeAg status or activity of liver disease, nor did she receive interferon therapy during the interval (Table 1).

In this study, core gene deletions were found in 6.5% of patients with chronic HBV infection. Although we did not sequence all the fast migrating bands, we have reasons to believe that these bands represent core gene deletions rather than PCR artefacts or contaminants. All ten fast migrating bands that we sequenced had core gene deletions. Additional band(s) were detectable in the follow-up samples from eight of ten who had multiple bands initially, but in only three of 119 patients who had single bands initially.

The fastest migrating bands for all six patients from whom virus core genes were sequenced had deletions/mutations that resulted in smaller or truncated core proteins. These changes may generate defective genomes since core protein is essential for nucleocapsid assembly, pregenome encapsidation and HBV DNA synthesis (Birnbaum & Nassal, 1990; Yu & Summers, 1991; Nassal, 1992). *In vitro* studies have shown that C-defective viruses can replicate by *trans*-complementation from wild-type virus as well as C+ defective genomes.
(Horwich et al., 1990; Okamoto et al., 1993). We found that all the patients with smaller band(s) on the ethidium-bromide-stained gels had a band of the expected size. Sequence analysis revealed that the bands of the expected size from these patients had intact pre-core/core gene except for a few amino acid changes suggesting that these sequences can provide trans-complementation to the defective genomes.

In accordance with other investigators, we found that the major deletions involved the centre of the core gene (Okamoto et al., 1987; Wakita et al., 1991). Four of six patients had in-frame deletions of codons 79–122. This region harbours a major CD4+ T cell and two B cell epitopes. It is possible that deletions of these epitopes may help to evade immune clearance thus favouring persistence of virus infection. Although the co-existence of these defective genomes together with intact sequences suggest that infected hepatocytes may still be able to express HBe epitopes, in vitro studies have shown that the expression of wild-type HBeAg can be decreased by the concomitant presence of defective core proteins (Horwich et al., 1990).

The other two patients had identical frameshift deletions which resulted in the loss of the first nine codons of the overlapping P gene. Naturally occurring P-gene-defective HBV has been reported (Blum et al., 1991), but is uncommon. This is probably related to the important replicative functions of the P gene products. In addition, while P-defective genomes can replicate with trans-complementation, the complementation is less efficient compared to S- or C-defective genomes (Okamoto et al., 1993). It is therefore interesting to note that both defective genomes had a potential start codon for the P gene exactly nine codons upstream of the major deletions. These two patients also had additional mutations that created a premature stop codon resulting in truncation of the core protein; otherwise translation of the core protein will continue till the end of the P gene because of the frameshift.

In eight of ten patients serially studied, smaller band(s) remained detectable in the follow-up samples. The persistence of these defective genomes supports the notion that they are replication competent with trans-complementation. Only three (3%) patients who had single bands initially had additional band(s) in the follow-up samples, suggesting that the development of viable, major deletions is uncommon. Although all three patients received interferon therapy during follow-up, the subsequent appearance of additional band(s) can be attributed to interferon therapy in one patient only (patient 20). The lack of additional band(s) in the follow-up samples of the other 71 patients who also received interferon therapy, and the presence of smaller band(s) in the initial samples of 17 patients prior to interferon therapy suggest that most deletions were not induced by interferon therapy.

Of the four patients who were serially studied, three had identical deletions in the follow-up samples and very few additional changes in the undeleted parts, indicating that these defective genomes were stable over a period of up to 5 years.

Contrary to Wakita et al. (1991), we found that core-gene-defective HBV can be detected in both asymptomatic carriers as well as in patients with chronic hepatitis. In addition, we found that patients with core-gene-defective HBV were not more likely to clear HBeAg than those with intact core genes. Interestingly, multiple bands were not detected in any of the 62 HBeAg-negative patients studied. This may be related to insufficient amount of helper virus in such patients.

In summary, we found that core-gene-defective HBV that resulted in smaller or truncated core proteins can be found in 8.5% of HBeAg-positive but in none of the HBeAg-negative patients with chronic HBV infection. All the patients had co-existent intact core genes. The persistence of these deletions suggest that the defective genomes are stable and that they may contribute to the chronicity of the infection. The deletions were predominantly located in the centre of the core gene with little or no changes in the overlapping P gene. We acknowledge that we do not have direct proof that the deletions result in defective genomes. We also recognize that we may have missed defective genomes that are incapable of exportation. We are currently in the process of cloning the HBV DNA from all samples with multiple bands so that we can characterize the intermediate bands and sequencing the intrahepatic HBV DNA from these patients to determine if additional deletions can be found in the liver. In addition, we will conduct in vitro studies to determine if co-infection of wild-type and core-defective genomes can down-regulate wild-type virus replication or wild-type virus protein expression.

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References


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