Rapid detection and further characterization of infection with hepatitis B virus variants containing a stop codon in the distal pre-C region

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Recently, hepatitis B virus (HBV) replication in the absence of HBe antigenaemia has been attributed to HBV variants with a TAG stop codon in the distal pre-C region associated with one or two point mutations. We describe here a rapid detection method for the diagnosis of such HBeAg-negative HBV variants using selective oligonucleotide hybridization. The entire pre-C region was amplified by the polymerase chain reaction and hybridized under stringent conditions with non-mutated (M0), one (M1) and two (M2) point-mutated oligonucleotide probes. Of the 15 HBeAg-positive (group I) and 20 HBeAg-negative (group II) serum samples studied, 14 samples in group I and one sample in group II hybridized with M0 only and 18 samples in group II hybridized with M1 or M2, or both. The remaining two samples (from groups I and II, respectively) failed to hybridize with any of the three probes. DNA sequencing confirmed mixed distal pre-C sequences in samples hybridizing with more than one probe and also revealed novel mutations in the distal pre-C region of the two samples which failed to hybridize with any of the probes. The latter sample had a +2 frameshift and hence represented a new type of HBeAg-negative HBV variant. This method may therefore prove useful in the diagnosis of infections by HBeAg-negative HBV variants resulting from common mutations in the pre-C region, as well as for the identification of less common variants with novel mutations in the same region.

Introduction

During the course of hepatitis B virus (HBV) infection, seroconversion from HBeAg to anti-HBe usually correlates with improvement of liver disease and elimination of circulating HBV particles. Continuing liver disease associated with circulating HBV virions has, however, been repeatedly found in a significant proportion of patients positive for anti-HBe (Matsuyama et al., 1985; Bonino et al., 1986). Recently, several independent reports revealed the presence of HBV variants containing a defective pre-C region in patients positive for anti-HBe (Matsuyama et al., 1985; Bonino et al., 1986). During the course of hepatitis B virus (HBV) infection, seroconversion from HBeAg to anti-HBe usually correlates with improvement of liver disease and elimination of circulating HBV particles. Continuing liver disease associated with circulating HBV virions has, however, been repeatedly found in a significant proportion of patients positive for anti-HBe (Matsuyama et al., 1985; Bonino et al., 1986). Recently, several independent reports revealed the presence of HBV variants containing a defective pre-C region in patients positive for anti-HBe (Matsuyama et al., 1985; Bonino et al., 1986; Carman et al., 1989; Brunetto et al., 1990; Okamoto et al., 1990; Tong et al., 1990). The mutation in the pre-C region explained the absence of HBeAg synthesis and secretion in this category of patients despite virus replication. Although several different types of mutation could be responsible, including mutation of the initiation codon and frameshift mutations (Okamoto et al., 1990; Tong et al., 1990), in more than 90% of cases this is caused by an in-frame TAG stop codon in the distal pre-C region (see Fig. 1). The significant proportion of patients suffering from chronic hepatitis due to these HBeAg-negative variants, and the unpredictable but often rapid progression of the disease, called for a rapid and reliable method for diagnosing such HBV variants. Technology available to many virological laboratories without resorting to DNA sequencing was crucial as specific interferon therapy protocols for treatment of this subset of patients have been developed (Brunetto et al., 1989). To this end, we have established a method using a combination of the polymerase chain reaction (PCR) and DNA dot hybridization techniques.

The theoretical background of this method lies in the fact that the distal pre-C region is highly conserved among the different HBV genomes so far sequenced (Galibert et al., 1979; Pasek et al., 1979; Fujiyama et al., 1983; Ono et al., 1983; Kobayashi & Koike, 1984; Bichko et al., 1985; Okamoto et al., 1986, 1988; Vaudin et al., 1988; Rho et al., 1989; Tong et al., 1990) and, compared with HBeAg-positive HBV genomes, HBV variants with a TAG stop codon are associated with either a single point mutation or two point mutations in the distal pre-C region (Carman et al., 1989; Brunetto et al., 1990; Okamoto et al., 1990; Tong et al., 1990) (see Fig. 1). Therefore, we have synthesized three 21-mer or 22-mer oligonucleotides, corresponding to non-mutated
and one or two point-mutated distal pre-C sequences, respectively. By amplification of the DNA sequence of the pre-C region followed by stringent hybridization with the three oligonucleotide probes (oligoprobes), mutation(s) in the distal pre-C region were easily detected and differentiated. Study of 20 HBeAg-negative cases confirmed and extended our previous findings of an inactive pre-C region in association with HBV replication, despite the presence of anti-HBe (Tong et al., 1990).

**Methods**

Serum samples and PCR amplification of the pre-C region. Included in this study were 15 HBe Ag-positive (group I) and 20 HBeAg-negative serum samples (group II) from chronic hepatitis B patients. Most of the patients in group II had chronic active hepatitis. Of these, three were negative for both HBeAg and anti-HBe antibody whereas the remaining 17 were positive for anti-HBe antibody. By dot hybridization, 12 samples in group I and two samples in group II were positive for HBV DNA. To extract DNA, 300 µl of serum was incubated at 70 °C for 1 h in the presence of 0.5% SDS and 2.5 mg/ml proteinase K (Bethesda Research Laboratories). After incubation, serum was extracted with phenol/chloroform, precipitated with ethanol and DNA was resuspended in 30 µl of H₂O.

Serum DNA (3 µl) was amplified in 100 µl of PCR solution containing 50 mM-Tris-Cl pH 8.3, 6 mM-MgCl₂, 1 mM-KCl, 1 mM-DTT, 0.4 mM of each dNTP, using 100 pm of each of primers pX30 and pC24. In the presence of 2.5 units of *Taq* DNA polymerase (Cetus Corporation), 35 cycles were performed. Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and chain elongation at 72 °C for 2 min. Cloned HBV genomes HBV-wt (Galibert et al., 1979) and HBV-wt (Galibert et al., 1979) were also amplified as controls. After amplification, 10 µl of PCR product was electrophoresed in a 2% agarose gel, using *@X174* replicative form DNA as a size marker.

Dot hybridization with oligoprobes. Three mutation detection oligonucleotides, M0 (non-mutated), M1 (one point-mutated) and M2 (two point-mutated), were synthesized (see Fig. 1). Using a DNA 3' end-labelling kit (Boehringer Mannheim), 30 pmol of oligonucleotide was labelled with 10 µl of [α-<sup>32</sup>P]dCTP (10 mCi/ml). The non-incorporated [α-<sup>32</sup>P]dCTP was removed by centrifugation through a Sephadex G-50 column.

Depending on the DNA yield, 5 to 10 µl of PCR product was denatured and dotted onto the nitrocellulose filter using a 96-well Hybri-Dot manifold apparatus. The filter was baked at 80 °C for 2 h. Prehybridization and hybridization were carried out in a sealed plastic bag. The filter was prehybridized for 2 h with a solution containing 6 x SSC, 10 x Denhardt's solution, 0.2% SDS, 100 µg/ml denatured and sheared salmon sperm DNA (ssDNA) and subsequently hybridized for 2 h with a solution containing 6 x SSC, 0.2% SDS, 100 µg/ml ssDNA and 2 x 10⁶ c.p.m./ml oligoprobes. After hybridization, the filter was successively washed once for 5 min in 6 x SSC/0.1% SDS, 2 x SSC/0.1% SDS and finally in 1 x SSC/0.1% SDS.

**DNA sequence determination.** The DNA sequence was determined from either PCR clones or directly from PCR products. The 400 bp DNA fragment was purified from a 1.5% low melting point agarose gel and digested with *EcoRI* and *BglII*. The resultant 210 bp *EcoRI*-*BglII* fragment, covering the entire pre-C region and part of the C gene, was cloned into M13mp18. When the *BglII* site was absent, the complete 400 bp fragment was cloned by the *EcoRI* and *HpaI* sites. Sequencing of cloned DNA has been described elsewhere (Tong et al., 1990). For direct sequence determination of the PCR product, ssDNA was generated by 35 cycles of asymmetric PCR. Using 1 pmol of pC24 and 100 pmol of pX30, from a previous conventional PCR, 5 to 25 ng of DNA was amplified. The ssDNA was purified by centrifugation through a Sephadex G-50 column.

Using 10 units of T4 polynucleotide kinase, 10 pmol of sequencing primer S21 (Fig. 1) was labelled at the 5' end with 2 µl of [α-<sup>32</sup>P]dATP (10 mCi/ml). Labelled primer was centrifuged through a Sephadex G-50 column, precipitated and resuspended in Tris-EDTA buffer. About 2 pmol of labelled S21 was annealed at 65 °C with single-stranded template DNA. DNA sequencing was performed by the Sanger chain termination method using a Sequenase kit (United States Biochemicals).

**Results**

Specific PCR amplification of the pre-C region sequence

A fragment of approximately 400 bp corresponding to the expected 391 bp from either cloned HBV DNA or directly from serum samples (Fig. 2) was visualized by ethidium bromide staining after 35 cycles of PCR. To confirm the molecular identity of the amplified fragment, the PCR product was digested with *BglII*, which cuts at nucleotide 1986 in most known HBV sequences. As expected, the DNA was digested, yielding two smaller fragments of 210 bp and 190 bp, respectively. These DNA fragments were Southern-transferred and probed with <sup>32</sup>P-labelled S21. Consistent with the locations of the *BglII* site and S21 sequence in the C gene.
Rapid detection of HBV variants

Fig. 2. PCR amplification of the pre-C/C gene from cloned DNA HBV-α1 (lane 1) and a serum sample (lane 3). The patterns after digestion with BglII are shown in lanes 2 and 4, respectively. Left, ethidium bromide staining; right, Southern blot hybridization with S21 probe. Size marker, φX174 DNA digested with HaeIII (lane M).

(Fig. 1), S21 hybridized with the 400 bp fragment and the smaller (190 bp) BglII fragment only (Fig. 2).

Establishment of stringent hybridization and washing conditions for the mutation detection probes

Since the consensus sequences M0, M1 and M2 differed from each other by only one or two nucleotide changes, stringent hybridization and washing conditions were crucial to the success of the mutation detection method. To set up sensitive and specific hybridization conditions, cloned HBV DNAs with no mutation (HBV-wt), one point mutation (HBV-γ2) and two point mutations (HBV-α1) in the distal pre-C region were used. The effect of combining different hybridization and washing temperatures (Tm = 15 °C, Tm = 5 °C) on the detection specificity and sensitivity was investigated. The Tm value was calculated by the formula Tm = 2 × A/T + 4 × G/C (Meinkoth & Wahl, 1984). In agreement with other reports (Conner et al., 1983; Sano et al., 1988), we achieved sensitive yet specific results by hybridizing at Tm ≈ 5 °C and washing at Tm ≈ 5 °C, or by carrying out both hybridization and washing steps at Tm = 5 °C (data not shown). These latter conditions were adopted for all subsequent experiments and the specific hybridization results of the three probes are shown in Fig. 3(a).

In order to detect simultaneously one and two point-mutated pre-C sequences which both had a TAG stop codon, we combined equal amounts of M1 and M2 probes in the hybridization procedure. Since the Tm value for both M1 and M2 was 68 °C, hybridization and washing were carried out at 63 °C. Fig. 3(a) shows that the combined M1 + M2 probe hybridized with HBV-α1 and HBV-γ2, but not with HBV-wt.

Detection of mutated pre-C region sequences from patients' serum samples

Using cloned HBV DNA, with the stringent hybridization conditions set up, we tested for possible one or two point mutations in the pre-C region from 20 HBeAg-negative samples. As controls, 15 HBeAg-positive samples were included. Replicate filters were hybridized with M0 (Fig. 3b), M1, M2 and the combined M1 + M2 probe (Fig. 3c). The results are summarized in Table 1. Of the total 35 samples, one HBeAg-positive (B3) and one HBeAg-negative (E2) sample failed to hybridize with any of the three probes. The remaining 14 HBeAg-positive samples and 1 HBeAg-negative sample (E2) failed to hybridize with any of the three probes. The remaining 18 HBeAg-negative samples, eight hybridized with more than one probe, including M0 + M1, M0 + M2 and M1 + M2 (see Table 1). Of the

Table 1. Hybridization results with different oligoprobes

<table>
<thead>
<tr>
<th>Oligoprobe</th>
<th>HBeAg+</th>
<th>HBeAg-</th>
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<tbody>
<tr>
<td>M0</td>
<td>14 (2)*</td>
<td>1 (1)</td>
</tr>
<tr>
<td>M1</td>
<td>0</td>
<td>5 (2)</td>
</tr>
<tr>
<td>M2</td>
<td>0</td>
<td>5 (2)</td>
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<tr>
<td>M0 + M1</td>
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<td>0</td>
<td>2 (2)</td>
</tr>
<tr>
<td>M1 + M2</td>
<td>0</td>
<td>5 (2)</td>
</tr>
<tr>
<td>None</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>15 (3)</td>
<td>20 (11)</td>
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* Samples for which the diagnosis had been confirmed by DNA sequencing.
three samples negative for both HBeAg and anti-HBe antibody, one hybridized with M2 (G1), one with M1 and M2 (G2) and the other with M0 only (F4).

Confirmatory DNA sequencing of the pre-C region

To confirm the diagnosis made by dot hybridization, the DNA sequence was determined from PCR products of several samples. Direct sequencing of PCR products was adopted for most cases as it reflected combined sequences of all DNA molecules within a given sample. Sequencing data of the seven samples which hybridized with only one probe matched hybridization results. No mutation was found in the entire pre-C region of the anti-HBe antibody-negative sample F4, which hybridized with the M0 probe only. Direct sequencing of five samples that hybridized with more than one probe showed mixed pre-C sequences and confirmed the diagnosis obtained by using oligoprobes (Fig. 4).

The failure of samples B3 and E2 to hybridize with any of the three probes suggested that the distal pre-C region sequences in these samples were different from M0, M1 and M2. DNA sequencing revealed several point mutations in B3 when compared with our previously determined pre-C sequences (Tong et al., 1990). Within the oligoprobe region there was a single point mutation compared with the M0 sequence, changing the GGG codon at nucleotides 1888 to 1890 into a GGA codon (Fig. 4 and 6). The mutation was silent at the amino acid level, for both codons specified glycine. The pre-C region sequence of E2 has also been determined from PCR products and PCR clones. There was one adenine or cytosine insertion in the proximal pre-C region and another cytosine insertion in the distal pre-C region covered by the oligoprobes (Fig. 5 and 6). Thus, E2 represented a novel HBV variant with a +2 frameshift mutation in the pre-C region.

Discussion

In this report we have described a rapid oligonucleotide detection method to screen for common HBV variants containing a TAG stop codon in the distal pre-C region. This was feasible because of the high degree of sequence conservation among non-variant HBV and limited (one or two point) mutations associated with the most prevalent pre-C variants. Under appropriate conditions, the oligonucleotide probe is very specific and will not hybridize with a DNA sequence containing even a single
mismatch (Wallace et al., 1981). To overcome the low detection sensitivity inherent to the oligoprobe (Rakoczky et al., 1989), like others (Farr et al., 1988; Saiki et al., 1988) we turned to PCR technology in order to amplify the short DNA sequence flanking the mutated sites. The specific amplification of the pre-C/C gene was verified by enzymic digestion and hybridization with an oligoprobe. Due to the exponential increase in the amount of DNA yielded by PCR, a 2 h hybridization followed by overnight exposure was usually sufficient to obtain interpretable results.

To detect the mutations in the distal pre-C region, 3 oligonucleotides were synthesized. The region that we chose was highly conserved among all 14 HBV sequences available (see Introduction for references). The fact that 33 of the 35 samples studied hybridized with at least one of the three probes further confirmed strict sequence conservation in this part of the pre-C region. To facilitate the simultaneous detection of one and two point-mutated pre-C variants (both had a nonsense mutation), we synthesized oligonucleotides M1 and M2 with different lengths but identical Tm values. As the oligoprobes identified only the nonsense mutation in the distal pre-C region, HBeAg-negative samples that hybridized with M0 only had to be sequenced to look for potential mutations in the upstream pre-C sequence. On the other hand, samples containing an M1 or M2 sequence in the distal pre-C region invariably had an inactive pre-C region. This remained true, even in the case of an additional nucleotide insertion, deletion or point mutation occurring in the upstream sequence.

Hybridization results of 20 HBeAg-negative cases revealed a nonsense mutation in the majority of them. Samples from our study were equally divided between those containing one and two point mutations, whereas a two point mutation was more prevalent in Greece (Carman et al., 1989) and a one point mutation in Japan (Okamoto et al., 1990). Also of interest was the identification of a mutation in the pre-C region from two of the three cases negative for both HBeAg and anti-HBe. Whereas most HBeAg-negative samples hybridized with one probe only, some samples did hybridize with more than one probe. Direct sequencing of PCR products confirmed the presence of mixed pre-C sequences in these samples. In addition, we have cloned PCR products of several such samples into M13 and found different pre-C clones within a single sample (data not shown). By cloning the complete HBV genome we have previously found different HBV clones (one point-mutated and two point-mutated) circulating in the same patients (Tong et al., 1990). Carman et al. (1989) have detected in some HBeAg-negative patients mixed populations of non-mutated and one point-mutated pre-C sequences. Since it has been suggested that the pre-C region mutations may arise under anti-HBe immune pressure (Okamoto et al., 1990), the presence of mixed pre-C sequences may represent a stage when only some molecules in the virus pool have undergone mutation or when different molecules exhibited different mutations. An application of our detection method will therefore be to monitor the proportion of mutated and non-mutated genomes during the natural history of HBV infection, but it should be used primarily to monitor treatment with antiviral compounds.

The two samples (one HBeAg-positive, the other HBeAg-negative) which failed to hybridize with the three probes turned out to contain, within the oligoprobe region, either a one point mutation or a one nucleotide insertion. Due to a frameshift mutation, E2 represented a novel HBV variant unable to synthesize HBeAg. This further demonstrates that the hybridization conditions used are highly specific and do not tolerate even a single nucleotide mismatch or insertion. Furthermore, these findings illustrate the utility of the method reported here for identifying less frequent HBV variants with novel mutation(s) in the pre-C region. Samples that do not hybridize with M1 or M2 are potential candidates for such novel HBV variants.

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References


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