Hepatitis B virus genomes from long-term immunosuppressed virus carriers are modified by specific mutations in several regions

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There is increasing evidence that hepatitis B virus (HBV) infection of an immunosuppressed host is associated with the appearance of virus mutants. To characterize the virus circulating in patients in detail, eleven full-length HBV genomes, isolated from the serum of five highly viraemic renal transplant recipients with liver disease, were cloned and sequenced. The genomes contained deletions in the C gene, deletions in the pre-S1/2 region frequently removing the pre-S2 initiation codon, premature termination codons in the pre-S1 or S region, and/or deletions/insertions in the X gene/core promoter. The mutations occurred at different positions and in various combinations; even mutant genomes circulating within a patient differed strikingly. It is concluded that long-term immunosuppression is associated with the occurrence of heterogeneous populations of partially defective HBV characterized by a specific mutation pattern. Efficient intracellular trans-complementation probably enables high virus replication in vivo.

A large variety of mutations has been identified in hepatitis B virus (HBV) genomes (Günther et al., 1999). Specific mutations, namely deletions in the C gene (Günther et al., 1996a), deletions/insertions in the core promoter that generate novel hepatocyte nuclear factor 1 (HNF-1) sites (Laskus et al., 1994a; Günther et al., 1996b; Pult et al., 1997) and deletions in the pre-S1/2 region (Trautwein et al., 1996; Pult et al., 1997) appear to accumulate in particular in HBV from immuno-suppressed patients. In some patients, accumulation of these variants is associated with severe liver disease (Günther et al., 1996a; Trautwein et al., 1996; Bock et al., 1997; Pult et al., 1997). Therefore, it is conceivable that the above-mentioned mutations, particular combinations thereof or as yet unidentified mutations in other regions of the genome are associated with a reduced immune response and may even play a role in pathogenesis. However, so far HBV genomes from only one immunosuppressed heart transplant recipient who experienced liver failure have been completely sequenced (Pult et al., 1997); subgenomic sequences have been analysed in all other studies. Therefore, we have investigated the structural features of eleven full-length HBV genomes (A–K) isolated from the serum of five renal transplant recipients with severe liver disease (biochemical, clinical and/or ultrasonographic signs of liver cirrhosis). The patients were positive for hepatitis B s antigen (HBsAg) and hepatitis B e antigen (HBeAg), and were both HBV-infected and treated with different combinations of immunosuppressive drugs (methylprednisolone, cyclosporin A and/or azathioprine) for periods between 7 and 14 years. None of the patients had evidence of infection with human immunodeficiency virus, hepatitis C virus (tested by PCR and immunoassay) or hepatitis D virus. One patient was positive for hepatitis G virus by PCR. All patients were highly viraemic at the time of sampling as determined by hybridization assay (1–3 determinations per patient; mean, 19–6 ng HBV DNA/ml serum; range, 3–100 ng/ml).

Serum HBV DNA was purified by proteinase K digestion and phenol–chloroform extraction. Full-length 3·2 kb HBV genomes were amplified by PCR as described previously (Günther et al., 1995) using primers P1/P2 (P1, HBV nucleotides 1821–1841, CCGAAAAGCTTGAAGCTCCCTCTTTTACCTCTGCTAATCAA; P2, 1823–1806, CCGAAAAGCTTGAAGCTCCTTCAAAAAAGTTGCAATGTTGCTGG; heterologous sequences to facilitate cloning are underlined). In order to roughly map regions with length heterogeneity, the PCR products of the full-length PCR were used as templates for subgenomic PCR with primers P1/P3,
Fig. 1. For legend see page 2689.
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amplified full-length genomes were digested with confirmed and extended by sequencing. To this end, the observed in three regions: C gene, pre-S1 wild-type fragment. Length heterogeneity, as indicated by 1505–1527). Subgenomic amplicons were separated in 1394–1372; P12, 1266–1286; P13, 1620–1599; P14, 1505–1527). Subgenomic amplicons were separated in ethidium bromide-stained gel and compared with an HBV wild-type fragment. Length heterogeneity, as indicated by aberrant bands in addition to the band of wild-type length, was observed in three regions: C gene, pre-S1/2 regions and 3′-end of the X gene that overlaps the core promoter region (data not shown).

The results of the structural analysis by PCR were confirmed and extended by sequencing. To this end, the amplified full-length genomes were digested with SsI within the heterologous primer sequences and cloned into vector pUC19. From every patient, one to three cloned HBV genomes (in total eleven genomes) were completely sequenced using vector- and HBV-specific primers (see above). The results of the sequencing can be seen in detail in Fig. 1 and common mutations are summarized in Table 1. Consistent with the PCR analysis, eight of the eleven genomes contained deletions in the C gene, ten had deletions in the pre-S1/2 region and ten had deletions/insertions in the core promoter/X gene. All deletions in the C gene were in-frame, which predicts expression of internally truncated core and pre-core proteins. Seven of the eight C gene deletions were located upstream of the P gene ATG, whereas one deletion also affected the N terminus of the P gene, which encodes the priming domain. According to previous experiments, the former deletions at least are likely to render the genomes defective for autonomous replication (Okamoto et al., 1993; Yuan et al., 1998b). Similarly to the C gene deletions, all deletions in the pre-S1/2 region were in-frame, which predicts production of pre-S1 and/or pre-S2 protein with internal deletions, provided their expression is not prevented by additional mutations (see below). Simultaneously, these deletions shortened the spacer domain of the virus polymerase and removed part of the pre-S2/S gene promoter. Four genomes contained pre-S1/2 deletions which remove the pre-S2 start codon and thus prevent expression of the pre-S2 protein. Deletions concerning exclusively the pre-S1 region or the pre-S2 region were observed in two and four genomes, respectively. In four genomes, expression of a full-length pre-S1 protein was prevented by mutations generating premature termination codons at positions 75 or 77 of the pre-S1 region. Premature termination codons at positions 95, 182 or 216 of the S region preventing expression of full-length pre-S1, pre-S2 and/or S protein were found in six genomes. Altogether, none of the eleven genomes had the coding capacity for full-length pre-S1 protein; only one had the coding capacity for full-length pre-S2 protein; and only five had the coding capacity for full-length S protein. A further hot spot for mutations was the 3′-end of the X gene/core promoter region. In this region, ten genomes were affected by three different types of mutations, namely by duplications of upstream regulatory sequences of the core promoter and by short insertions or deletions, occasionally accompanied by nucleotide changes, in the basic core promoter. As has been demonstrated recently by protein–DNA binding assays, mutations in the basic core promoter, as found in genomes B, C and E–K, create novel transcription factor binding sites for HNF-1 (Günther et al. 1996b), whereas the insertion in genome A creates a sequence motif with similarity to the binding motif of HNF-3. Simultaneously, nearly all mutations in the core promoter led to a frameshift in the X gene, which predicts expression of C-terminally truncated X protein. Phylogenetic analysis revealed that all genomes belong to genotype A. Compared to the genotype A consensus sequence, a variable number of nucleotide and amino acid differences was found per genome. However, there were only two common hot spots for amino acid changes, namely positions 142/143 of the core protein (threonine-142 to arginine; leucine-143 to proline or isoleucine) and positions 7/8 of the polymerase (histidine-7 to glutamine or aspartate;
A variety of mutations has already been identified in HBV from chronically infected, immunocompetent patients. For example, mutations in the pre-C region preventing expression of HBeAg and specific amino acid changes in the C gene as well as in the immunodominant B cell epitope of HBsAg (a-determinant) were frequently observed (Günther et al., 1999). HBV with these mutations often emerge in patients who develop antibodies to HBeAg (Okamoto et al., 1990; Carman et al., 1997) or HBsAg (Kato et al., 1996), which may indicate that the B cell and/or T cell response plays a role in their selection. The mutations common to genomes from immunosuppressed patients are infrequent in HBV genomes from immunocompetent patients (Günther et al., 1999). Deletions in the C gene and deletions/insertions in the core promoter were even found to disappear upon seroconversion to anti-HBe in immunocompetent patients (Laskus et al., 1994b; Marinos et al., 1996), but were selected under immunosuppressive conditions (Laskus et al., 1994a; Günther et al., 1996a; Pult et al., 1997). This epidemiological pattern does not point to a major role of the immune response in their selection. Altogether, the molecular epidemiological data suggest that two major sets of mutations, resulting from adaptation of the virus either to a host who immunologically responds to the infection or whose immune response is suppressed, occur in HBV.

Table 1. Summary of common mutations in HBV genomes from immunosuppressed patients

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genome* Patient†</th>
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<tbody>
<tr>
<td>Deletion in C gene</td>
<td>8 5</td>
</tr>
<tr>
<td>Deletion in pre-S1/2 region</td>
<td>10 4</td>
</tr>
<tr>
<td>Deletion/insertion in core promoter/X gene</td>
<td>10 5</td>
</tr>
<tr>
<td>Premature stop codon in pre-S1 region</td>
<td>4 4</td>
</tr>
<tr>
<td>Defect in pre-S2 protein expression</td>
<td>4 3</td>
</tr>
<tr>
<td>Premature stop codon in S region</td>
<td>6 3</td>
</tr>
<tr>
<td>Amino acid change in P gene (position 7/8)</td>
<td>8 5</td>
</tr>
<tr>
<td>and C gene (position 142/143)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of genomes out of a total of 11 in which the mutation occurs.
† Number of patients out of a total of 5 in which the mutation occurs.
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References


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