Clinical relevance of mutations in the precore genome of the hepatitis B virus

H Tillmann, C Trautwein, D Walker, K Michitaka, S Kubicka, K Böker, M Manns

Abstract

A stop codon in the precore genome of the hepatitis B virus (HBV) in anti-HBe positive HBV carriers may be associated with a more progressive form of HBV infection. Earlier studies, however, were mainly performed in patients from the Mediterranean area who had severe infection. The aim of this study was to evaluate the prevalence of precore mutants in an unselected population living in northern Europe. Twenty of 42 of these patients are infected predominantly with a virus strain, which has the typical stop codon in the precore genome, characterised by a mutation at base 83. In six patients there was an additional G to A mutation at base 86 of the precore genome. Statistical analysis showed no difference between the patients with or without a stop codon in the precore genome. When patients with a double mutation at base 83 and 86 of the precore genome were compared with the other anti-HBe positive HBV carriers, however, the corresponding clinical data were worse. Therefore we suggest, that it is not the stop codon in the precore gene itself, but the occurrence of a double mutation at bases 83 and 86, which is associated with a more severe course of disease in anti-HBe positive HBV carriers.

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Hepatitis B virus (HBV) infection can lead to a wide variety of clinical manifestations, ranging from acute self limited illness to different forms of chronic infection progressing to liver failure in some patients. The ordinary HBV induces production and secretion of HBsAg and HBeAg particles in the infected hepatocytes. HBeAg, derived from sequences in the core and precore region of the viral genome, is mainly produced during active replication and production of entire viral particles. Its determination is therefore used as an indirect marker of virus replication. Recently, however, it has been shown that high viral replication may occur without circulating HBeAg and even in the presence of circulating anti-HBe antibodies. This phenomenon was mainly seen in patients with a severe course of disease.¹ When rapid sequencing methods became available, it was possible to show that this can be caused by mutations that prevent the HBV from producing HBeAg. 'Stop codon' and 'frame shift' mutations within the precore gene, or deletion of the precore initiation codon

(ATG) have been described.²⁻¹⁷ The most commonly seen mutation is a G to A change at base 83 of the precore gene leading to the formation of a stop codon (TAG). Several reports suggested that this precore mutation is associated with a poor prognosis in chronic hepatitis B²⁻¹¹ and may even be associated with an increased incidence of fulminant hepatic failure.¹²⁻¹⁴ However, this mutation was also found in asymptomatic carriers.¹⁵⁻¹⁷

The prevalence and clinical relevance of the base 83 precore HBV mutant seems to differ geographically. As most studies of precore HBV mutants originated in southern Europe we studied the prevalence of precore mutants in our regional population of northern Germany. We evaluated the clinical significance concerning the severity of liver disease in patients with the HBV precore mutation in a laboratory screened population, irrespective of their ethnic background and their disease activity. Additionally, we used a simple and quick method to detect mutations of base 83 of the precore genome, which may serve as a routine screening method.

Methods

Patients

A total of 2460 serum samples were screened for viral hepatitis between 1 January 1992 and 30 September 1992 in our laboratory, which serves as a reference laboratory. The patients were referred to our university hospital by their general practitioners or from smaller hospitals.

The prevalence of precore mutants was evaluated in the anti-HBe positive population of HBV carriers. The ethnic background was analysed for those patients whose samples could be sequenced.

Hepatitis virus serology

Serological diagnosis was performed for HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBe, anti-HDV, and anti-HCV (second generation) with commercial enzyme linked immunosorbent assay (ELISA) tests according to the instructions of the manufacturer (Abbott Laboratories, Chicago).

Quantitative analysis of HBV-DNA in the serum samples was performed with a commercial radioactive hybridisation assay (Abbott Laboratories, Chicago).

DNA extraction

DNA was digested with proteinase K for two

Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, Germany
H Tillmann
C Trautwein
D Walker
K Michitaka
S Kubicka
K Böker
M Manns

Correspondence to:
Professor Dr M P Manns,
Department of
Gastroenterology and
Hepatology, Zentrum Innere
Medizin und Dermatologie,
Medizinische Hochschule
Hannover, 30625 Hannover,
Germany.

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TABLE 1 Primers for routine PCR, allele specific PCR, and sequencing

Pre-C-sense primer	GGA GGC TGT AGG CAT AAA TTG GTC
Pre-C-antisense primer	GAT CTT CTG CGA CGC GGC GAT TGA GA
Ordinary hepatitis B primer	(CCA AGC) TGT GCC TTG GGT GGC TTT G
Mutant primer	(CCA AGC) TGT GCC TTG GGT GGC TTT A
BC-I	GGA GGC TGT AGG CAT AAA TTG GTC

Parentheses show the six bases that were different between the long and the short primer for the PCR.

hours at 56°C, extracted by a standard protocol with phenol/chloroform/isoamylalcohol (25:24:1), and precipitated with ethanol. The pellet was resuspended in TE-buffer (10 mM TRIS, 1 mM EDTA) and either used immediately or frozen at -20°C.

Polymerase chain reaction (PCR) of the precore region
HBV-DNA precore/core sequence was amplified by PCR with synthetic oligonucleotides spanning the precore/core region. We used 30 pmol pre-C-sense primer (Table I) and 30 pmol pre-C-antisense primer, 10 pmol dATP, dCTP, dGTP, and dTTP each, 10 µl 10xTaq-buffer (500 mM KCl, 100 mM TRIS-Cl pH 8.4, 15 mM MgCl₂, 0.1% gelatine) 2,5 U Taq-polymerase (Gibco, BRL, Eggenstein, Germany) in 100 µl total volume. After an initial two minute denaturing step at 93°C we ran 35 cycles, each lasting one minute at 93°C, one minute at 50°C, and one minute at 72°C. After the last cycle there was an additional five minutes at 72°C to complete elongation. The PCR product was electrophoretically visualised in an ethidium-bromide stained 2% agarose gel.

Sequencing
PCR products from the ordinary HBV-PCR were isolated with glass milk (Dianova) and sequenced with the dideoxynucleotide chain termination method¹⁸ using ³⁵S labelled ATP and BC-I Primer (T7 Sequencing Kit, Pharmacia Biotech, Freiburg, Germany; ³⁵S-ATP, Amersham Buchler, Braunschweig).

Allele specific PCR
We also established an allele specific PCR for the point mutation at base 83 of the precore genome. Therefore we used two different sense primers ending at base 83 of the precore

sequence and corresponding either to the ordinary HBV (83G) or to the HBeAg-minus mutant (83A) and a common pre-C-antisense primer, which is also used in the routine PCR method. The PCR protocol differed in a very high annealing temperature of 75°C or 72°C depending on the primers' length (Table I).

Clinical data and statistical analysis
Laboratory data were obtained for all patients of whom HBV sequencing data had become available. The Wilcoxon's rank test was used for calculation of significant differences.

Results

Stop codon and frame shift mutations in the precore genome of the anti-HBe positive HBV carriers
Two hundred and forty one of all patients screened for viral hepatitis in a nine month period were HBsAg positive and of these 127 patients were anti-HBe positive. HBV-DNA was detected by PCR in the serum in only 68 (54%) of 127 anti-HBe positive carriers. In 26 of 68 patients the amount of HBV-DNA was too low for the less sensitive allele specific PCR or a sequencing reaction. Therefore results were available for allele specific PCR and sequencing reactions in 42 of 68 patients. Nineteen (45.2%) patients were predominantly infected with the HBV mutant at base 83 of the pre-C-genome, while 22 (52.3%) patients were infected predominantly with the ordinary HBV. One patient had the mutant and ordinary HBV in almost equal amounts, shown by similarly strong bands for G (guanine) and A (adenine) in the sequencing reaction at base 83 of the pre-C-genome (Fig 1). Ten of 30 patients of German origin exhibited the mutated HBV while 10 of 12 patients of foreign origin showed the mutation at base 83. Five of 10 immigrants with this mutation came from Turkey, one from Saudi Arabia, one from Russia, one from Greece, one from former Yugoslavia, and one from Italy. Among the 22 patients who were infected with the ordinary HBV at position 83, one patient of Polish origin showed a C to T mutation at the 4th base of the precore genome, which leads to a stop codon at the second codon. Therefore 21 (50%) of all patients had a stop codon in the precore open reading frame of their predominant or codominant HBV strain. Two patients of German origin had an insertion of an A (adenine) between base 24 and 27 (TA(A)AT) of the precore gene leading to a frame shift mutation. Thus 23 of 42 (54.7%) patients had precore genome sequences unable to produce HBeAg. The only patient of foreign origin with no mutation preventing HBeAg secretion was a dentist, who probably became infected while working as a dentist in Germany.

Other mutations in the precore genome of anti-HBe positive HBV carriers
Beside the mutations preventing the HBV from producing HBeAg, seven other mutations

Figure 1: Sequence at base of the potential stop mutation of the precore genome. (A) mutant virus only; (B) codominant infection with mutation at base 83 and ordinary HBV; (C) mutation of base 83 and 86; (D) ordinary HBV infection only. C=cytosine; T=thymine; A=adenine; G=guanine.

Figure 3: HBV-DNA concentrations in relation to base 83 and anti-HBe status. Data shown as mean (SD).

patients with the double mutation *v* *p*=0.0249 for the patients with the ordinary HBV and the patients with the mutation at base 83 only). All patients of this group show signs of active liver disease compared with the other patients included in our study; those with a single mutation of base 83, or those belonging to HBV genotype A. Seven of 14 patients (50%) infected with the first and seven of 14 patients (50%) infected with the second virus type are asymptomatic carriers.

Discussion

In our study, the occurrence of HBeAg-minus mutations, characterised by the introduction of a stop codon at base 82–84 is less frequently found compared with Italian or eastern European studies where nearly all the patients who are anti-HBe positive seem to have this

mutation.^{6 20} The lower rate of these mutations in our region becomes even more evident, when patients with foreign ethnic background are excluded. This striking difference may partly result from the higher prevalence of the genotype A in the German population compared with Italian populations. This is supported by the fact that all patients with genotype A in our study were of German origin. The genotype A is characterised by a cytosine instead of a tyrosine at base 45 of the precore genome. This base is exactly opposite base 83 in the putative hairpin structure of the pregenomic HBV-RNA.²¹ Therefore a cytosine at base 45 stabilises TGG and prevents the mutation to TAG at bases 82–84. As the clinical relevance of genotyping for hepatitis C is under intensive investigation^{22–24} it is noteworthy that the patients with HBV genotype A seem to show clinical parameters of less severe liver disease than those with any other HBV genotype.

The age when people become infected with HBV may be important for the development of HBeAg negative mutants. In areas with high rates of perinatal/neonatal infection like southern Italy, high rates of precore mutants seem to be evident. HBeAg is presumed to play a part in the induction of immunotolerance towards HBV in the newborn.^{25 26} Therefore repression of HBeAg secretion in people infected at a very young age might be advantageous for HBV to survive the immune attack against the HBV due to age, while in persons infected at an older age only the suppression of viral replication is of any benefit.

In contrast with other studies, which investigated a preselected population for the occurrence of the HBeAg-minus mutation, we investigated the prevalence in an unselected population and related our epidemiological data to the clinical state. This may be one explanation why we could not find a correlation between the precore mutant at base 83 and more severe liver disease caused by HBV infection. It is intriguing, however, that we found a significant difference in the clinical pattern, when an additional mutation at base 86 was found in patients with the stop codon at base 82–84. From our data we would suggest that only the occurrence of both mutations is associated with a more severe course of HBV infection in these patients. We present evidence that the difference between those patients who only exhibit a mutation of base 83 and those with the additional mutation of base 86 seems to be important as only the double mutation correlates with an advanced stage of

TABLE III Clinical data for patients with a mutation of base 83 *v* the patients with the ordinary HBV

	Base 83 mutant		Ordinary hepatitis B virus	
ALT	60.16 (66)	NS	49.25 (52.99)	
AST	36.47 (27.33)	NS	40.15 (36.02)	
γ-Globulin	21.11 (9.06)	NS	23.25 (9.79)	
CHE	3.77 (1.84)	NS	4.05 (1.79)	
PTT	79.58 (20.21)	NS	84.72 (19.18)	
Bilirubin	30.52 (36.47)	NS	21.21 (21.77)	

Abbreviations as in Table II. Data shown as mean (SD).

TABLE IV Clinical data for patients with a mutation of base 83 and 86 *v* the patients with base 83 mutation and those with the ordinary HBV

	Base 83 mutant without base 86 mutant		Base 83 and 86 mutation		Ordinary HBV	
ALT	54 (70.23)	NS	73.5 (59.43) <i>p</i> =0.08*	NS	49.25 (52.99)	
AST	29.31 (23.02)	NS	52 (31.55) <i>p</i> =0.07*	NS	40.15 (36.02)	
γ-Globulin	18.08 (6.02)	<i>p</i> =0.043	27.67 (11.52)	NS	23.25 (9.79)	
CHE	4.24 (1.74)	NS	2.72 (1.77)	NS	4.05 (1.79)	
PTT	87.08 (13.86)	<i>p</i> =0.025	63.33 (23.37) <i>p</i> =0.014*	<i>p</i> =0.023	84.72 (19.18)	
Bilirubin	21.46 (24.64)	NS	50.17 (51.52) <i>p</i> =0.061*	NS	21.21 (21.77)	

Abbreviations in Table II. Data presented as mean (SD).

*Significance value for patients with the double mutation *v* patients with the ordinary HBV and patients with the mutation of base 83.

TABLE V Patients' age in relation to mutations of base 83 and base 86

Patients with double mutant	49.83 (10.36) years
Patients with ordinary HBV	44.86 (16.95) years
Patients with base 83 mutant	41.28 (11.42) years

Data shown as mean (SD).

disease. Thus our data do not support the hypothesis that the mutation of base 83 in the precore genome is directly responsible for the progressive course of the disease in anti-HBe positive HBV carriers. However, this phenomenon could also just be a marker of a more advanced disease. It may be that the double mutation at base 83 and 86 occurs in patients with longer duration of their disease as those exhibiting the double mutation tend to be of older age (Table V).

We analysed only those patients whose serum had adequate amounts of HBV-DNA for direct sequencing. We are aware that this could form some selection bias. It is known, however, that anti-HBe positive patients' serum exhibiting the mutation at base 83 show higher amounts of DNA compared with patients' serum with ordinary HBV⁶ and it is believed that the mutation at the base 83 is correlated with a worse prognosis.²⁻¹¹ We realise that it would also be interesting to analyse the HBV-DNA in liver biopsy specimens of these patients. As there was no clinical necessity, however, no biopsy specimens were taken for scientific reason.

We would favour the hypothesis that the mutation at base 83 of the precore sequence is a marker for associated mutations such as the mutation of base 86 of the precore sequence, which are associated with a progressive form of disease. Base 86 already belongs to the Kozak sequence of the core gene m-RNA.²⁷ The Kozak sequence is part of the sequence just upstream of the initiation codon and influences ribosomal translation of the specific mRNA.²⁷ Although a G to A mutation in this Kozak sequence may influence the expression of the core gene, it could be speculated that a quantitative change in the presentation of the core peptides on the cell surface may lead to an increased attack of the immune system against the infected hepatocytes. Therefore further investigations are needed to support the hypothesis that additional mutations besides the stop codon at base 82-84 are responsible for the more progressive course of disease among these patients, and whether this may lead to an enhanced expression of the core protein.

There are 12 possible mutations in the precore region, which would create a stop codon by a single nucleotide change. However, only two of these mutations are found in our study. The mutation described in the second codon, a C to T mutation at the 4th base seems to be less important in our population as this variation was found in only one patient of Polish origin. The most frequent mutation, similar to many other studies, was the G to A change at base 83 of the pregenome. Beside these two mutations and the insertion of an adenine

between base 24 and 27 in two additional patients, no other mutation disabling HBeAg secretion was found in our population. We did not find mutations of the initiation codon, although five different possibilities have been described.^{15 20 28 29} These mutations sometimes represent only minor virus populations,^{15 28} which may not be found by direct sequencing.

It is still unknown why the potential stop codons appear only in so few of the possible codons. As this region harbours different functions of the virus, however, such as nuclear encapsidation, viral replication, and binding sites for transcription factors it is also probable that these changes would interfere with the normal life cycle. The double repeat region 1 is essential for viral replication³⁰ and spans base 10 to base 21 of the precore gene, therefore it is not surprising that mutations are not found in this region. The last 54 bases of the precore gene were shown to belong to the encapsidation signal.^{21 31} Tong *et al* were able to show that two possible mutations (TGC-to-TGA, bases 67-69; TGG-to-TGA, bases 76-78) within the encapsidation signal²⁰ producing stop codons show a defect in RNA packaging and might therefore not exist in nature.³²

HBeAg is not found in some patients despite viral replication, although there is no mutation preventing the HBeAg production on the DNA level. In these patients additional mechanisms may exist – outside the precore region.

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