J.Cell.Mol.Med. Vol 6, No 1, 2002 pp. 113-143

Special Article

Molecular, immunological and clinical properties of mutated hepatitis B viruses

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Received: February 25, 2002; Accepted: March 6, 2002

Abstract

Hepatitis B virus (HBV) is at the origin of severe liver diseases like chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma. There are some groups of patients with high risk of generation of HBV mutants: infected infants, immunosupressed individuals (including hemodialysis patients), patients treated with interferon and lamivudine for chronic HBV infection. These groups are the target for molecular investigations reviewed in this paper. The emergence of lamivudine- or other antiviral-resistant variants, rises concern regarding long term use of these drugs. Infection or immunization with one HBV subtype confers immunity to all subtypes. However, reinfection or reactivation of latent HBV infection with HBV mutants have been reported in patients undergoing transplant and those infected with HIV. Mutations of the viral genome which are not replicative incompetent can be selected in further course of infection or under prolonged antiviral treatment and might maintain the liver disease. Four open reading frames (ORF) which are called S-gene, C-gene, X-gene and P-gene were identified within the HBV genome. Mutations may affect each of the ORFs. Mutated S-genes were described to be responsible for HBV-infections in successfully vaccinated persons, mutated C-genes were found to provoke severe chronic liver diseases, mutated X-genes could cause serious medical problemes in blood donors by escaping the conventional test systems and mutated P-genes were considered to be the reason for chemotherapeutic drug resistance. This paper reviews molecular, immunological and clinical aspects of the HBV mutants.

Keywords: hepatitis B virus - genome - mutations - immunity - liver disease

Introduction

Hepatitis B virus is worldwide spread with most cases in Africa and Asia but also in the Mediterranean countries [1, 2, 3]. It can be transmitted parenterally (blood products/needle sharing), sexually, perinatally or by saliva.

During the last years numerous mutations of the HBV genome have been described. The mutation rate of the HBV genome was calculated to $2x10^4$ base substitutions per site per year [4 and ref. herein]. Only mutations which are not replicative incompetent and which are associated with a functional change get clinical importance. For example mutated virus strains can be the reason for HBV-infections in successfully vaccinated persons [5, 6, 7 and ref. herein], for the failure of interferon [8, 9, 10] or lamivudine [11] therapy and may be at the origin of severe liver diseases like chronic active hepatitis [12], liver cirrhosis [13, 14, 15] and hepatocellular carcinoma [14, 16, 17, 18].

This paper reviews the molecular, clinical and immunological properties of the HBV mutants.

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Genomic organisation and gene products of the hepatitis B virus

Hepatitis B virus is a double-stranded DNA virus, the prototype of the family Hepadnaviridae. Its highly compact genome (3182 bp) contains four major open reading frames (ORFs) encoding: the envelope (preS1, preS2 and HBsAg), the core, the X protein and the polymerase. The S-gene (NT 2848 to NT 833; type ayw_3) codes a 226 aa protein, named major antigen. The preS2 gene codes a 55 aa protein, which together with the previous major antigen form the medium antigen. The preS1 gene codes a 108 aa protein which together with the previous medium antigen form the large antigen. The C-gene (NT 1814 to NT 2450) codes the core-protein (HBcAg) which is a part of the viral nucleocapsid or the HBe antigen which is secerned into the blood, respectively. The X-gene (NT 1374 to NT 1836) has various gene-regulating functions and the P-gene (NT 2307 to NT 1621) codes the viral polymerase (Fig. 1).

Replicative cycle and immunological features of HBV infection

HBV has an interesting replicative cycle using an RNA intermediate. After infection of the hepatocyte the HBV-DNA is transferred to the nucleus where the plus-strand is completed. Thereafter mRNAs encoding for the different viral gene products and the pregenomic RNA are transcribed. Now the viral proteins are translated by the ribosomes and the pregenomic RNA is encapsidated. First the DNA-minus-strand is synthesized by reverse transcription from the pregenomic RNA. Thereafter the pregenomic RNA is degraded and the DNA-plus-strand is synthesized. The ripe virion passes the cell membrane where it gets its envelope.

HBV has no direct cytopathic effect and liver damage is caused by immunological features. Viral proteins expressed on hepatocytes are targets for T-cells. T-cell-mediated immune response is the main mechanism for virus clearance in acute self-limited hepatitis.

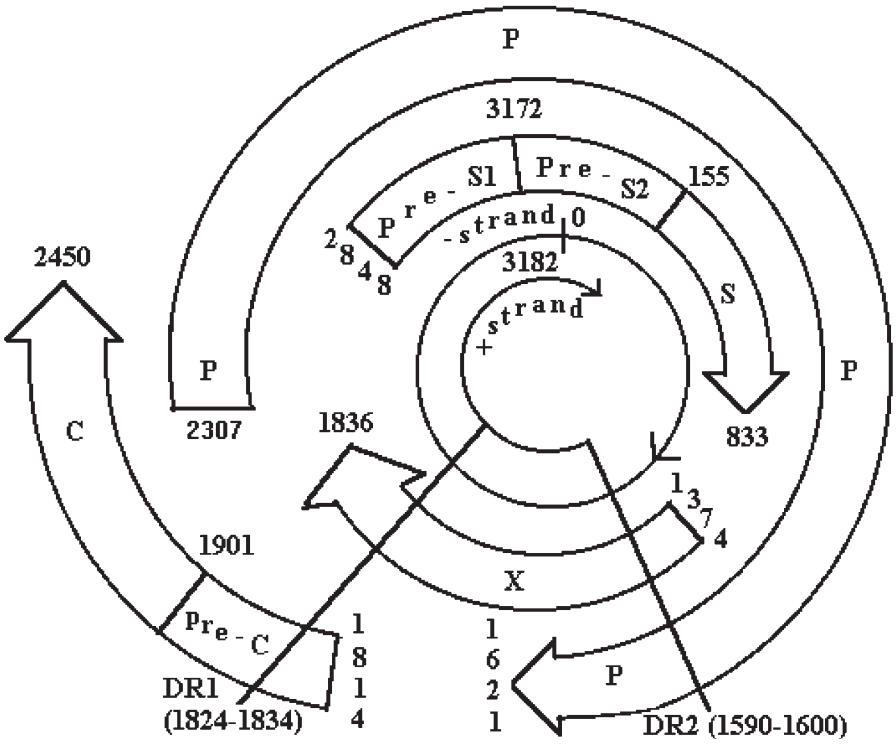

Fig. 1 Gene map of the HBV genome (subtype ayw3). The numbers at the beginning of the genes indicate the first nucleotide of the gene. The numbers at the end of the genes indicate the first nucleotide which follows the gene. The start of the unique EcoRI site was designated as nucleotide 0 = nucleotide 3182. The first and the last nucleotide of the direct repeat sequences DR1 and DR2 are shown.

The S-gene

The S-gene contains of 3 regions and encodes for 3 different glycoproteins which only differ in the length of their N-terminus. The gene products are designated the s (small)-HBsAg (or major-HBsAg) (226 aa) - encoded only from the S-region -, the m (medium)-HBsAg (281 aa) - encoded from the pre-S2-region and the S-region - and the l (large)-HBsAg (400 or 389 aa depending on subtype) - encoded from the pre-S1-region, the pre-S2-region and the S-region [20].

Mutations of the small-HBsAg

Inside the S-gene the different alleles a, d, y, w (four subdeterminants w_1 to w_4 are described), r and q can be distinguished [21 and ref. herein]. The determinants d/y as well as w/r are mutually exclusive. This leads to at least 9 serotypes of the small-HBsAg. The a-determinant which is part of all HBs-subtypes can be divided into two alleles. They only differ in one amino acid (126 t = Thr, 126 i = Ile) [22]. The main subtypes are therefore designated ayw, ayr, adw and adr.

Their geographical distribution was found to be stable over two decades. The subtype ayw was described in the Mediterranean countries (ayw_2, ayw_3), in West- and Central-Africa (ayw_4, ayw_2) and in Vietnam (ayw_1). The rare subtype ayr is found in Vietnam. The subtype adw is predominant in North-West-Europe, in America, in East and South Africa, in India (adw_2) and in South-East Asia (adw_4). The subtype adr can be found in Polynesia ($adrq^-$) and in South-East-Asia ($adrq^+$) [23, 24].

The clinically most important determinant of HBsAg is the a-determinant (aa 124 to 147). During the natural course of infection antibodies against all determinants are produced [ref. in 25] but only the antibodies against the a-determinant are protective against a challenge with other HBV subtypes. Mutations within the a-determinant could be

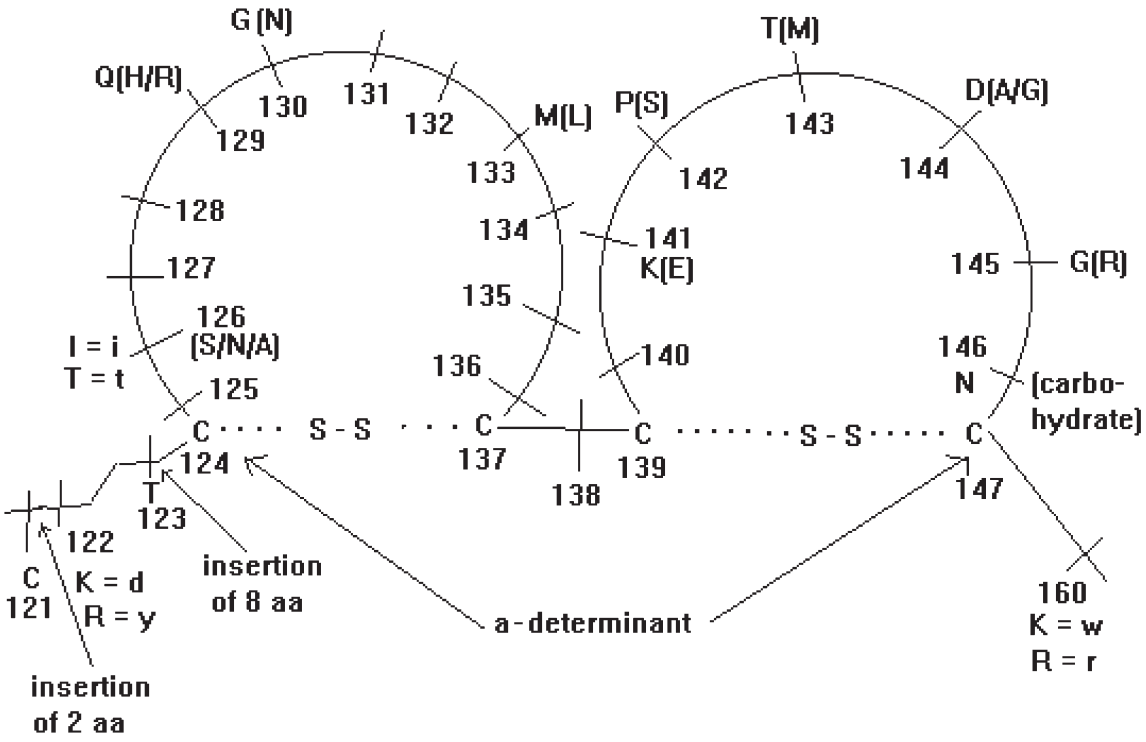

Fig. 2 The loop structure and possible mutation sites of the a-determinant. The aa are indicated in the one letter code. The disulphide bridges are marked by punctated lines. The new aa created by point mutations are shown in parenthesis.

selected for example after a successful active or passive vaccination.

The conformation of the a-determinant is a two loop structure which is stabilized by disulphid bridges between Cys-124 and Cys-137 (first loop) and Cys-139 and Cys-147 (second loop) [26] (Fig. 2). The 4 Cys at the basis of the two loops were found to be highly conserved [4]. If this conformation is altered, priviously produced antibodies against the native a-determinant are no more protective [5]. This could last in an infection of a sucessfully vaccinated person or in a reinfection of a former HBV infected patient [27, 28, 29, 30].

Mutations which abolish the two loop structure of the a-determinant are changes in the hydrophilicity, the electrical charge or the acidity of the loops (Pro-127 to Ser [31], Gln-129 to Asn [32] or Leu [33], Thr-131 to Ile [32], Met-133 to Thr [34], Phe-134 to Ser [35], Lys-141 to Arg [35], Pro-142 to Ala [36], Thr-143 to Met [37], Asp-144 to Ala [38, 39], Gly [40] or Glu [27], Gly-145 to Ala [41, 42], Gly-130 to Asp [43] or Glu [44], Cys-138 to Arg [44], Pro-142 to Ser [38], Gln-129 to His [38, 45] or Arg [29], Met-133 to Leu [38], Gly-145 to Arg [5, 38, 46], Ile/Thr-126 to Asn, Ser [47] or Ala [29], Thr-131 to Asn [48], Lys-141 to Glu [49], Asp-144 to Val [36], Asn-146 to Thr [50] or Asp [42]). Furthermore additional possible N-linked glycosylation sites (Gly-130 to Asn [6]), binding of a carbohydrate moiety to Asn-146 or changing the stability of the disulphid bridge (Cys-147 to Gly [50]) could explain changes of the two loop conformation. Mutations nearby the a-determinant (insertion of 8 aa between Thr-123 and Cys-124 [4, 47], of 2 aa between Cys-121 and Lys-122 [4, 51, 52], or of Arg between Pro-120 and Cys-121 [27] as well as aa changes (Asn-116 to Thr [53], Val-118 to Ala [53], Pro-120 to Ser [53, 31], Cys-121 to Phe [50], Ala-159 to Val [53], Phe-183 to Cys [53], Val-184 to Ala [53])) might also disturbe the secondar structure of the a-determinant.

Mutations which do not touch the a-determinant but only change the other alleles w to r (Lys-160 to Arg [6]) do not have clinical importance.

Interestingly the allelic mutation d to y (Lys-122 to Arg [54]) could be correlated with a higher failure rate of passive-active immunoprophylaxis in infants of HBeAg positive mothers [55, 56].

Mutations between codon 40 and 49 [27, 57] and between codon 198 and 208 [27] that do not alter the a-determinant were found in patients with immune globulin prophylaxis after orthotopic liver transplantation. The mutations within the first region could be selected by immune pressure because this region was found to contain a major histocompatibility class I-restricted T-cell epitope [58]. The significance of the second region remains unclear.

Mutations of the large-HBsAg and of the medium-HBsAg

The medium-HBsAg (pre-S2) and the large-HBsAg (pre-S1) are very important for the virus clearence because they are more immunogenic and appear earlier in the course of infection than the small-HBsAg. Vaccines with all the three HBsAg were found to cause higher antiHBs titers than vaccines with the small-HBsAg alone [59, 60]. Recently a A to G change at NT 2794 within the promotor of the large-HBsAg was found [36]. This mutation lead to a decreased protein expression and it may be speculated that immunological properties were altered.

The pre-S1-region contains two important epitopes. One of them (aa 58 to 100) is speculated to be recognized by antibodies which are involved in viral clearance. The other one (aa 21 to 47) seems to be the hepatocyte binding region. A deletion of the antibody recognized region which do not touch the hepatocyte binding size could inhibit viral clearance [25, 47, 61, 62].

Deletions within the pre-S1 region can eliminate the promoter of the small-HBsAg. This kind of mutations was found in patients with persistent infections. The large-HBsAg that was encoded by these variant genomes was non-functional and the virus maturation was stopped. However the small-HBsAg from the wildtype virus was sufficient to restore the generation of infective HBV particles [63].

The N-terminus of the pre-S2-protein was described to bind specifically to the fibronectin found in the human liver sinusoids. The pre-S2-protein therefore might be involved in the viral attachment to host tissues [64].

Surprisingly most aa encoded by the pre-S2-region seem to be dispensable for the viral life cycle.

The aa-sequence 109 to 163 of the large-HBsAg is encoded by the pre-S2-region. While the aa 109 to 113 were found to be indispensable, deletions spanning the domain between aa 114 and 163 could be tolerated [65, 66].

Deletions which affected the initiation-codon of the pre-S2-mRNA caused an entire loss of the medium-HBsAg. The observation that the HBV easily tolerates these mutations might allow the conclusion that m-HBsAg is not necessary for the viral life cycle. Pre-S2 defective viruses were described in chronic carriers after the seroconversion to antiHBe-positive [67] and in HBV-reinfected patients after orthotopic liver transplantation [40]. It therefore was speculated that the loss of the highly immunogenic pre-S2-protein is a mechanism to escape the immune system [4, 6, 47, 61].

HBV variants with changed S-genes were described in the European low prevalence areas like the UK [28], Germany [62] and France [64] and also in the high prevalence area of the Mediterranean countries [5]. Moreover they were found in the USA [27], in Brazil [45], in the Southern Africa [37], in the South-East-Asia [29, 38, 35] and in Japan [47]

The C-gene

The C-gene contains of two regions. The core-region (183 aa) encodes for the viral nucleocapsid (HBc-antigen) and the precore-region (29 aa) together with the core-region for the HBe-antigen which is secerned into the blood. After being processed at its N-terminus and its C-terminus the ripe HBe protein generally contains of the aa 20-29 of the precore-sequence and of the aa 1-149 of the core-sequence [2 and ref. herein, 68, 69 and ref. herein, 70]. An other HBeAg which in addition to the normal HBe-sequence contained the aa 1-19 of the precore-region could be detected [70].

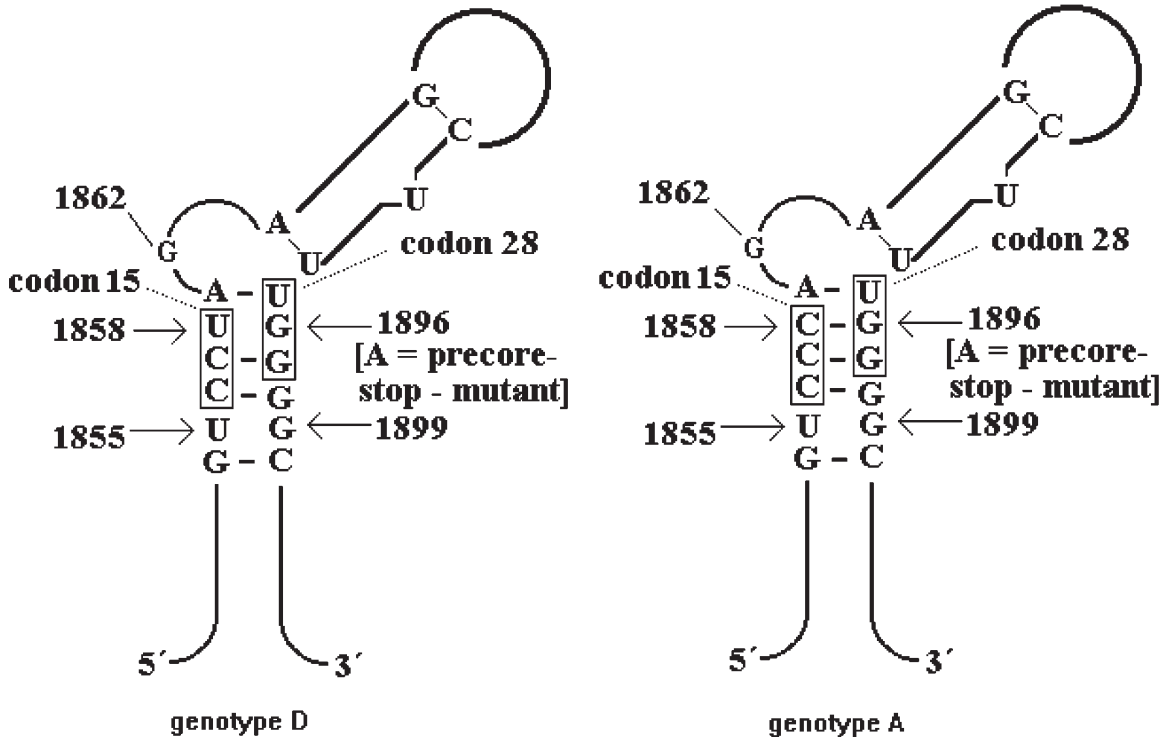

Fig. 3 The hairpin structure of the encapsidation signal of the pregenomic RNA. A part of the nucleotide sequence of the pregenomic RNA with the precore-codons 15 and 28 is indicated for the genotype D (left) and for the genotype A (right).

Mutations of the precore-region

The precore-region plays an important role in viral replication because the pregenomic RNA forms a hairpin structure (NT 1855 to 1858 pair with NT 1896 to 1899 (Fig. 3)) which binds the polymerase. This protein-RNA interaction is considered to be the first step of encapsidation of the pregenomic RNA into the nascent core particle. Furthermore the reverse transcription to minus strand DNA was found to be initiated within the hairpin structure [71, 72]. Therefore this hairpin structure is highly conserved.

For understanding why mutations within the encapsidation signal may affect the virus replication in a various manner it has to be mentioned that the HBV genome was found to exist in different genotypes which are distributed in various geographical regions. Seven prototype variants (A, B, C, D, E, F, and G) could be defined [73, 74, 75]. Their nucleotide sequences differ in at least 8%. Compared to genotype A, the genotypes B, C, D, E, F, and G have a 6 nucleotide-deletion at the C-terminus of the core-gene, in genotype G 36 nucleotides are inserted in the N-terminus of the core-gene [75], while genotype D has a 33 nucleotide-deletion at the N-terminus of the pre-S1-region, and genotypes E and G have a 3 nucleotide-deletion at the N-terminus of the pre-S1-region [76]. The type A could be found in the USA, in the North-West-Europe and in the sub-saharan Africa. The types B and C were found in the far east. The type F showed a high divergence and was speculated to be the original strain in the new world. The type D was found in the whole world while the origin of the type E was considered to be in West Africa [45 and ref. herein, 73, 77, 74]. The recently discovered type G was found in France and in the USA [75].

The observation that genotype A could be changed into genotype D after the seroconversion to antiHBe-positive [67] and vice versa [78] might be of great interest for future investigations.

Fig. 3 shows a part of the nucleotide sequence of the hairpin structure of the encapsidation signal. Genotype D is presented on the left and genotype A on the right. In genotype D a G to A mutation at NT 1896 stabilizes the hairpin structure by creating a U-A pair which might be advantageous for viral replication [71, 79]. This mutation changes the precore-codon 28 (Trp) into a stop codon and therefore prevents the expression of HBeAg. It was found in more than 95% of the precore-mutations [2, 3, 80, 81]. Strains of the genotype A however in which the stop-codon 28 mutation destabilizes the hairpin structure need an additional C to U mutation at NT 1858 to stabilize the encapsidation signal [82].

Mutations at the precore codon 17 (NT 1862 G to T) [83] seem to alter the processing of the HbeAg precursor protein. This might lead to a HbeAg negative phenotype.

Mutations at the precore codon 29 (NT 1899 G to A) have been found alone [84, 85] or together with other precore mutations like a G to T transversion at NT 1862 [86] or the 28-stop-codon-mutation [80]. They could be associated with HBeAg seronegativity but the mechanisms how these mutations lead to a HBeAg negative HBV-infection without further mutations still remains unclear.

Precore-mutations were found in all forms of infection from asymptomatic carriers [87, 88] to chronic active [85, 89, 90, 91 92] and fulminant hepatitis [92, 93, 94, 95, 96, 97, 98]. They could not be correlated with the severity of the liver disease or the forming of a chronic or a fulminant hepatitis [3, 80, 99, 100, 101]. Patients with orthotopic liver transplantation for HBV-related cirrhosis who were infected with precore-mutants before transplantation were considered to have the same prognosis as those who carried the wildtype virus at the same time [102].

It has to be mentioned that HBV-DNA can be integrated into the hepatocyte chromosomes. Interestingly HBV-DNA from liver tissues especially from hepatocellular carcinoma cells was found to possess more mutants than HBV-DNA from the serum [103, 104, 32]. An increasing number of mutations during integration could be at the origin of a functional change of the genome and therefore explain why these mutations frequently are associated with hepatocellular carcinoma but do not appear in the serum. An increasing number of precore-mutants such as 28-stop-codon mutations, deletions of the precore-start-codon (NT 1814) and/or frame-shift-mutations [103, 104] and point mutations in the a-determinant of the HBsAg [32] from acute

to chronic hepatitis to HBV infected non-tumour tissues and finally to hepatocellular carcinoma cells recently was discovered. The aa changes of the a-determinant Gln-129 to Asn or Thr-131 to Ile [32] or a G to A mutation at NT 1898 of the precore-region [104] exclusively were found in hepatocellular carcinoma cells but not in sera. The later mutation destabilizes the hairpin structure as in genotype A as in genotype D. Therefore it may be disadvantageous for viral replication and consecutively could facilitate integration of HBV-DNA.

Finally, also complete sequence identity of the precore-region could be observed between HBV-DNA from liver biopsies and from the serum but this study evaluated only 9 patients [105].

The evolutionarily highly conserved precore-Cys-23 seems to be crucial for the forming of solubile HBeAg or for the forming of particles. The presence of this Cys in the aa sequence of the ripe HBeAg was found to inhibit the particle agglutination thus allowing the HBeAg to be secerned into blood. An experimental change of precore-Cys-23 to Gln led to the formation of particles with HBc antigenicity [69].

The core-Cys-61 within the ripe HBeAg is the only Cys residue which in experiments was found to be able to form a Cys-61-Cys-61 disulphid bridge with another HBeAg. The resulting HBe-homodimers possess the antigenicity both of HBeAg and HBcAg [106]. However these HBe-dimers only are formed in the absence of precore-Cys-23. The experimental change of the Cys-61 led to a protein with HBe antigenicity only, but this antigenicity and the secretion efficiency strongly were reduced [107]. These experiments might allow the conclusion that mutants with additional Cys or change of the Cys-61 can alter the conformation of the HBeAg or the HBcAg. The newly developed proteins could no longer be recognized by antibodies [12, 107].

The precore-Cys-23 was found to be highly conserved in Taiwanese hepatomas [16] while in Italian hepatomas this Cys could be changed [108, 109].

HBV particles with mutations within the precore-region were described in the Mediterranean countries [2, 82, 85, 95, 110], in the USA [81], in Germany [84], in South-East-Asia [3] and in Japan [79].

Mutations of the core-region

The translation within the C-gene can start at the precore start codon or at the start codon of the C-region. If the ribosomes begin to translate at the precore start-codon they translate through the encapsidation signal. Thereby a recognition of the hairpin structure is not possible and the growing polypeptide HBeAg is lead to the secretion pathway. If the translation starts at the start-codon of the C-region, the encapsidation sequence is recognized and the developing HBcAg is encapsidated [111].

Various functional domains within the core-protein could be distinguished. For the self-assembly of the virus capsid the 144 aa at the N-terminus of the core-protein are sufficient [112, 113]. The arginine-rich C-terminus (up to aa 164) enables the encapsidation of the pregenomic RNA [113] and seems to stabilize the capsid by protein-nucleic acid interactions [112]. The remaining C-terminus (up to aa 173) may be important for the synthesis of plus-strand-DNA and thereby for the virus replication [113].

Furthermore the different epitopes within the core-protein differ in immunological aspects (see Table 1).

HBcAg and HBeAg are highly cross-reactive at the T-cell level [121, 122]. It therefore may be speculated that HBeAg presents immunogenic epitopes to the T-cells thus protecting the HBcAg expressing hepatocytes against the immune system. After the selection of a HBeAg-negative mutant the epitopes of the HBcAg might come under the pressure of the immune system [123].

Variants within the core epitopes in Spanish patients were found to be significantly correlated with precore-mutations that prevented the expression of the HBeAg [82]. However in Japanese patients core mutations could not be correlated with the appearance of precore-stop-mutations [124]. This might be due by different host factors.

During the asymptomatic period of infection the virus replication is reduced by CD_8^+ cytotoxic T-cells (CTL) whose target is the HBcAg. Mutations inside the CTL-epitopes of the C-gene therefore might create immune escape mutants leading to chronic viral persistence and severe liver disease.

A recent investigation [125] seems to confirm this opinion *in vitro*. The authors found that epitope 18 – 27 specific CTL that were isolated from

Table 1 Immunological aspects of the epitopes of the core – protein.

aa - sequence	Biological properties	Reference	Degree of conservation (cited from 12)
141 – 151	recognized by cytotoxic T – lymphocytes	114	very low
18 – 27	recognized by cytotoxic T – lymphocytes [CD_8^+ T – cells] HLA class I restricted	115, 116	high
1 – 20	recognized by HLA class II restricted T – cells [CD_4^+ cells]	117	very low
48 – 69			low
117 –131			high
84 – 101	recognized by cytotoxic T – cells	118	hot spot for deletions
74 – 83 (two overlapping domains)	conformational determinant responsible for HBc antigenicity	119	low
76 – 89	linear determinant responsible for HBe antigenicity	119	low
107 – 118	determinant responsible for HBc antigenicity	120	
around aa 138 (but aa 10 to 140 required for antigenicity)	conformational determinant for HBe antigenicity	119	low

patients with acute hepatitis poorly recognized mutations in this epitope. Epitope 18 – 27 specific CTL that were isolated from chronic hepatitis cases recognized some variations of the 18 – 27 epitope. This finding might be explained by the growing number of mutations during the time of infection. The growing number of mutations could lead to different CTL populations.

Naturally occurring aa-changes inside the CTL-epitopes were found in South-Eastern Asia [126, 127], in Japan [118], in Italy [128] and in Cuba [129] (Ser-21 to Val [126], His [129], Ala [128] or Asn [128], Phe-24 to Tyr [126], Ile-27 to Val [126] or Ala [128], Leu-84 to Ala [126] or Val [118], Ser-87 to Asn [126] or Gly [118, 126, 127], Tyr-88 to His [118, 126], Val-91 to Ala [118] or Ser [127], Met-93 to Thr [118], Gly-94 to Arg [127], Leu-95 to Ile [127], Lys-96 to Thr [127], Ile-97 to Leu [118, 126, 127], Gln-99 to His [126], Leu-100 to Ile [127], Leu-101 to Ile [118], Thr-147 to Ala [126, 127], Arg-151 to Gln [126, 127] or Cys [127]). However host factors also might play a role because an American investigation speculated the immune response of cytotoxic T-cells in chronic hepatitis not being strong enough to select mutants [130]. In chronic hepatitis the cytotoxic T-cell epitopes were no strongerly mutated than in patients who successfully cleared the virus. In the concept of these investigators the viral persistence in chronic liver disease is mainly due to a slow beginning of

the immune response which allows a great number of hepatocytes to be infected. Thereafter an entire virus elimination would no more be possible ever under enhanced immune pressure.

A sufficient HBe/HBc specific CD_4^+ T-cell activity is also considered to be required for an efficient immune response. The loss of the relevant epitopes for example by the prevention of the expression of HBeAg could be another immune escape mechanism. This concept may be confirmed by the fact that HBeAg specific CD_4^+ T-cells which did not recognize HBcAg were found in a chronic patient who only was infected by a HBeAg negative mutant [9].

The appearance or the selection of point mutations within the epitopes recognized by CD_4^+ T-cells also could prevent the immune response. This might lead to a chronic liver disease and thereby predispose for tumour formation. This hypothesis seems to be confirmed by the observation that variant viruses where aa-changes clustered within the CD_4^+ T-cell epitopes became predominant during acute exacerbations in chronic carriers [131].

Point mutations within the CD_4^+ T-cell epitope aa 1-20 [117] were described in hepatoma tissues from Taiwanese patients. Interestingly these mutations mainly were found at the highly evolutionarily conserved codons 5 (Pro to Thr), 13 (Val to Ala, Leu, Met or Gly) and 15 (Leu to Ser) [16].

Point mutations within the CD_4^+ T-cell epitope aa 48-69 [117] were described in severe liver diseases from patients from South-East-Asia [126, 127], from immunocompromized patients [132] and from hepatoma tissues [16] (Cys-48 to Val [126, 127], Ser-49 to Thr [126] or Glu [127], Pro-50 to Ala [126] or Ser [126], Leu-55 to Ile [126, 127], Ala-58 to Gly [127], Ile-59 to Val [126], Gly [127], Thr [16] or Phe [16], Leu-60 to Val [16, 126, 127], Gly-63 to Asn [127] or Val [16], Glu-64 to Asp [132], Leu-65 to Val [132], Met-66 to Thr [126], Thr-67 to Asn [132]).

Point mutations between aa 74 and 89 (Asn-74 to Ala [132], Glu-77 to Gln [129], Pro-79 to Gln [132], Asp-83 to Glu [129], Ser-87 to Thr [129]) may reduce both HBe antigenicity and HBc antigenicity. The change of Pro-79 to Gln [132] is of special interest because this Pro is highly conserved [133]. These mutations were found after antiHBe seroconversion in chronic active hepatitis and in immunocompromized patients.

Mutations at the codon 130 (Pro to Thr or Ser) might affect the cellular and the humoral immunity because this codon is part of a domain recognized and by B-cells [134] and by T-cells [117]. Interestingly the Pro to Thr mutation at the codon 130 frequently was found to be associated with a mutation at the codon 97 (Ile to Leu) [16]. One possible explanation for this phenomena could be the fact that the acquisition of Leu at codon 97 enables the HBV to secrete excessive amounts of virions containing incomplete single-strand DNA [45, 135]. This excessive secretion of incomplete virions can be offset by an additional mutation at codon 130 (Pro to Thr) [136].

Frame-shift-mutations and in-frame deletions that truncated the core-protein could be observed in immunocompromized patients [36] and in chronic carriers [131]. Two frame-shift-mutations shortened the corresponding core-proteins to 29 aa or to 65 aa, respectively [36], and an in-frame deletion caused a loss of the codons 94 to 101 [131]. In-frame deletions usually spanned various regions between the codons 80 to 130. Shortened core-proteins may be non functional. However functional core-proteins seem to be required for the nucleocapsid assembly. This would make the core-deletion-mutants dependent on the wildtype virus. The observation that these variants only were found together with the wildtype HBV [13, 137, 138] and transfection assays [36] could confirm this opinion.

Core-deletion-mutants mostly were observed in patients with long course infections. It was supposed that their selection may not be favorized by immunological features but by an enhanced expression of the polymerase. The over-expression of the polymerase is supposed to be inhibited by the two start-codons J (NT 2163 to NT 2165) and C2 (NT 2177 to NT 2179) which are situated within the C-ORF upstream of the normal polymerase-AUG (NT 2307 to NT 2309) [139]. Most core-deletion-mutants missed the inhibitory J-AUG and C_2-AUG whereas the polymerase-AUG was intact [13 and ref. herein]. Therefore it might be concluded that the appearance of core-deleted genomes led to a more efficient translation of the polymerase by the

ribosomes which might increase the intracellular polymerase level. This could result in an enhanced encapsidation of the pregenomic-RNA and thus in an increased virus production.

In immunocompromised patients core-deleted viruses can became preponderant over the wildtype virus. The decreased replication of the wildtype virus in these persons may be explained as follows. First, false core-proteins encoded by core-deleted HBV-variants could disturb the nucleocapsid assembly of the wildtype and the encapsidation of the pregenomic-RNA of the wildtype. This mechanism was observed *in vitro* [140] and *in vivo* [137]. Second, the core-proteins which are incorporated in the nucleocapsid of the mutants are no more available for the wildtype virus [13 and ref. herein, 15].

Although replication of the wildtype virus is decreased in immunocompromised patients, these patients generally were found to have high levels of viremia [141, 142]. This might be explained by an effective trans-complementation among partially defective viruses. It was shown *in vitro* that trans-complementation among partially defective viruses causes increased virus production [143]. In immunocompromised patients heterogeneous populations of partially defective HB viruses carrying deletions within the S-ORF, the C-ORF and/or the X-ORF were found together and it can be speculated that these genomes have an effective trans-complementation within the infected cell.

In immunocompetent patients the appearance of core-deletion-mutants was correlated with a low level of viremia [137]. This implies the predominance of other mechanisms regulating the virus replication than in immunocompromised persons.

The high concentration of polymerase molecules in hepatocytes which are infected with core-deleted HBV might lead to an increased degradation of the polymerase proteins and thereby more antigenic epitopes can be presented to the cytotoxic T-cells. Additionally non-functional core-proteins from core-deletion-mutants which are still able to interact with the functional core-proteins may create unstable core-hybrid-molecules. These hybrides could rapidly be degraded and then be presented to the immune system in a high quantity. Furthermore mutated core-proteins might show another antigenic structure than the wildtype protein that also would increase the number of determinants recognized by immunocompetent cells. Taken all together the appearance of shortened C-genes in immunocompetent patients could result in a selective disadvantage for the HBV [137].

Core-deletion-mutants could be associated with severe liver diseases like cirrhosis and necroinflammation in immunocompromised [13, 15, 141] and in immunocompetent patients [137].

In immunocompromised patients this observation might be explained by the disturbed nucleocapsid-assembly. The alteration of the normal virus genesis could result in the intracellular accumulation of HBV-specific products like HBcAg [144], HBsAg [145] or pre-S-proteins [146]. The overload of the hepatocyte with viral proteins may have direct cytotoxic effects.

The mechanisms leading to cytotoxicity in immunocompromised patients are caused by the virus and therefore should also exist in immunocompetent patients. Additionally in these cases the high quantity of antigenic determinants presented on the surface of the hepatocytes might result in a heavy cytotoxic activity of the immune system.

Finally, it has to be mentioned that the appearance of core-deletion-mutants in immunocompetent patients may reduce the replication of the wildtype HBV by the same mechanisms described in immunocompromised persons. This might result in a low level of HBeAg. It was speculated that a low HBeAg concentration could induce a reversible immune tolerance on the T-cell level [147, 148, 149]. Thereafter a HBcAg specific T-cell response might follow resulting in a necroinflammatory liver activity [137].

Interestingly the C-terminus of the core-protein (aa 150-171) seems to tolerate mutations easily in spite of its replicative functions [12]. The Ser-169 frequently was changed into Pro. This mutation was described in patients with fulminant hepatitis [133].

The influence of interferon treatment

Various investigations speculate that mutants which are unable to express HBeAg might be selected by the pressure of the immune system [84, 87, 150].

The following observations seem to support this hypothesis.

First, the 1896 G to A mutant accumulated in patients during interferon treatment [137]. Second, during interferon application an increasing number of precore-stop-codon-mutations like (NT 1817-1819 (CAA to TAA), NT 1874-1876 (AAG to TAG), NT 1895-1897 (TGG to TAG, TGA or TAA)) was observed while after the end of the therapy these mutants disappeared [84]. Third, a HBV strain in which the expression of HBeAg was already prevented by a mutated precore-start-codon (NT 1814) was described. This strain developed an additional precore-stop-codon under the pressure of interferon [8]. Fourth, HBe-negative mutants mostly appeared in patients with chronic hepatitis during the spontaneous or alpha-interferon induced seroconversion from HBeAg-positive to antiHBe-positive [84, 99, 151]. Fifth these variants could be associated with antiHBe positive hepatitis [100, 152, 153].

If the HBeAg-minus mutants are selected by immunologic features one should suppose that the appearance of these viruses is an immune escape phenomenon. The findings that precore-mutants were correlated with non-responsivness to interferon therapy [10] or with only a transient therapeutic effect [154] and that ongoing viremia and hepatitis reactivation during interferon therapy was associated with precore-mutants [150] may be in favour with this opinion.

However various observations contradict the hypothesis of an immune-mediated selection of the precore-mutants.

The reactivation of antiHBe positive hepatitis also was caused by the wildtype virus [152], small amounts of precore mutants were found together with the wildtype virus in most HBeAg positive carriers [100], asymptomatic chronic carriers with only the wildtype virus in their serum samples were described [155] and interferon did not always increased the number of precore-stop-mutations [152, 156]. Furthermore it has to be mentioned that HBeAg-positive Chinese patients with the 28-stop-codon mutation seemed to clear HBeAg more easily and to be more likely interferon responders than patients with the mutant Pro to Ser at codon 15 or patients with the wildtype virus alone [156]. In the cited study however the patients with the 28-stop-codon mutation had significantly higher levels of transaminases before treatment than the other groups which may be a favorable sign for interferon response [157].

Therefore an immune-independent mechanism leading to precore-mutants was postulated in addition to the immune-dependent mechanism.

It was found to be a poor therapeutic sign if, before interferon therapy was started, antiHBe-positive patients showed mutations within the core epitopes recognized by CD_4^+ T-cells or by B-cells [150]. One therefore might speculate that the variation of these epitopes is an immune escape mechanism and that viruses carrying mutated CD_4^+ T-cell or B-cell epitopes might be selected by the pressure of interferon. Interestingly the rate of mutations within the cited epitopes did not increase during the course of interferon treatment [150].

Finally it has to be mentioned that core-deletion-mutants were eliminated in patients with interferon therapy or with seroconversion to antiHBe positive [137]. This type of mutants could be correlated with a weak immune response in renal transplant patients [13] or in cases of chronic hepatitis acquired in the neonatal period [137].

The X-gene

The X-gene product HBx

The X-ORF encodes for a 154 aa protein called HBx. HBx was found to transactivate various cellular and viral genes with cis and trans activating elements [ref. in 158] and it may play a role in the development of the hepatocellular carcinoma [159, 160, 161, 162].

HBx can be divided into 6 domains (A-F). The C-terminus of the protein (domains C-E) seems to be the transactivating portion [158] while the N-terminus of HBx (domain A) can repress the HBx transactivation. Thereby the N-terminus is believed to avoid an excessive HBx transactivation and to play a role in a self-regulatory mechanism of X-gene expression [163].

The various domains of HBx show a very different degree of conservation among mammalian hepadnaviruses (see Table 2).

Interestingly the highly conserved domains A, C and E contain the 4 conserved cysteines 7, 61, 69 and 137 [158].

HBx is considered to be unable to bind directly to DNA [164, 165]. However an intrinsic

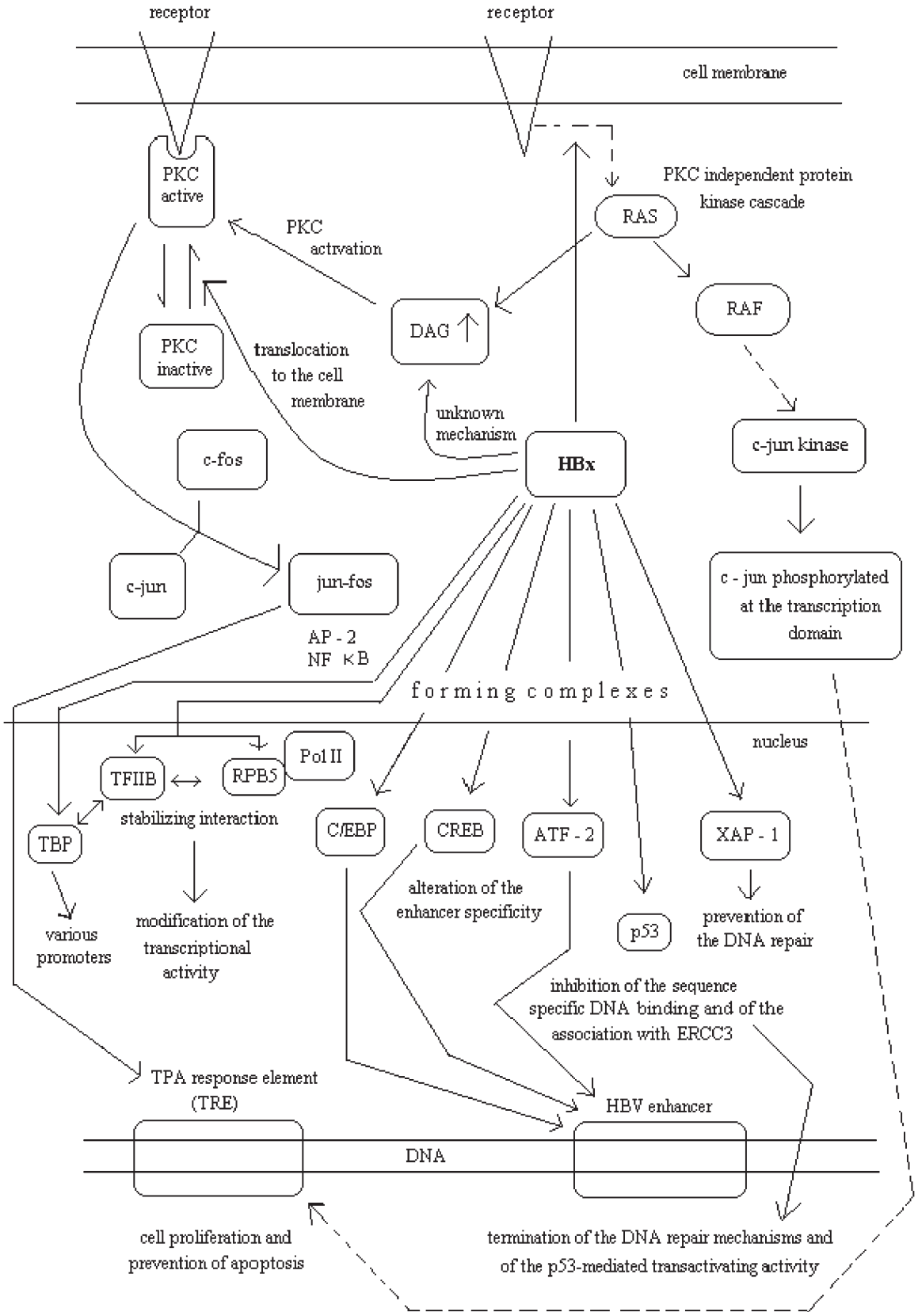

Fig. 4 Gene regulating pathways of HBx.

Table 2 Biological properties of the domains of HBx.

Domain	aa - sequence	Degree of conservation among mammalian hepadnaviruses	Importance for transactivation (cited from 121)
A	1 - 20	very high	not important
B	21 - 57	very low	not important
C	58 - 84	high	important but not crucial
D	85 - 119	low	not important
E	120 - 140	high	crucial
F	141 - 154	not conserved	not important

serine/threonine protein kinase activity could be detected [166]. This might imply that the transactivating activity of HBx depends on eukaryotic transcription factors.
HBx was found to act in the cytosol and in the nucleus. Various gene regulating pathways of HBx were described (Fig. 4).

Cytosolic pathways

In the protein kinase C (PKC) dependent cytosolic pathway HBx translocates the inactive cytosolic PKC to the membrane and thereby activates it [159]. Furthermore HBx increases the concentration of the endogenous PKC activator sn-1, 2-diacylglycerol (DAG) by an unknown mechanism. Thereafter the gene activation is carried out by the PKC dependent transcription factors c-jun/c-fos dimer (AP-1), AP-2 and NF κB.

In the PKC independent cytosolic pathway HBx activates the protein kinase cascade upstream of ras [167]. This pathway leads to the phosphorylation and thereby to the activation of the transactivating domain of c-jun.

Nuclear pathways

The transactivation domain of HBx was found to bind specifically to the central domain of the RPB 5 protein [168], a subunit which is common in eucaryotic RNA polymerases I-III. Additionally HBx can bind to the transcription factor (TF) IIB [169]. This results in a trimeric complex between RPB 5, TF IIB and HBx [170]. This mechanism may stabilize the normal interaction between TF IIB and the eucaryotic RNA-polymerase II. Thus the viral transactivator protein may selectively stimulate the promoters which interact with TF IIB and RPB 5.

Furthermore a direct specific interaction between the aa sequence 110 to 143 of HBx and the C-terminus of the TATA binding protein (TBP) was described [171]. The ability to form complexes with the TBP may explain why HBx can alter various transcriptional regulatory pathways and transactivate quite different promoters.

HBx by forming complexes with the nuclear transcription factors CREB or ATF-2 [172] may alter the enhancer specificity of these transcription factors. It was shown that HBx-CREB and HBx-ATF-2 bind to the HBV-enhancer whereas the transcription factors alone did not. Additionally HBx could bind to the DNA-binding domain of the C/EBP (CCAAT/enhancer binding protein) and thereby activate the HBV enhancer II in cis [173].

Protein-protein interactions may also be responsible for the oncogenic potential of HBx. HBx was found to bind the cellular anti-oncogene p53 in a complex. Thereby HBx may inhibit the sequence-specific DNA binding of p53 and block its transactivating activity. Furthermore the association of p53 with ERCC3, a transcription factor involved in nucleotide excision repair, was found to be inhibited by HBx [17]. This might explain that HBx can disturb the p53-ERCC3-mediated DNA repair.

The aa-sequence 55 to 101 of HBx was found to bind to XAP-1, a protein which normally is supposed to bind to damaged DNA thus initializing the nucleotide excision repair. This mechanism also might prevent an efficient cellular DNA repair [174].

The X-ORF overlappes the C-terminus of the P-gene and the N-terminus of the C-gene (see fig. 1). Depending on their extension mutations within the X-region therefore can affect three genes at once. X-deletions which created fusion-proteins between the polymerase and the core-protein (PC-proteins) or between the polymerase and a 3´ truncated HBx (PX-proteins) were found [175].

It might be expected that mutations within the X-ORF which affect the transactivating domain of HBx lead to a non replicative HBV. Investigations in the woodchuck model and clinical observations confirm this opinion. Woodchucks which experimentally could be infected with PC-proteins or with PX-proteins developed a non-productive liver disease without the serological markers [175].

Point mutations within the highly conserved domain E of the X-gene (Ile-127 to Thr, Lys-130 to Met, Val-131 to Ile) recently were described in Japanese patients with fulminant and chronic hepatitis [176] and in immunocompromized patients with fulminant hepatitis [132]. It was shown that these mutations affecting the crucial domain for transactivation suppress the transcription of both precore and core mRNA [177].

Frame-shift-mutations in patients which created a new stop-codon in the C-terminus of the X-gene led to a HBx which was 134 aa long. This protein had lost its transactivating activity and it resulted a stopped synthesis of the HBV pregenome and a hepatitis without the classical serological markers. 3` truncated X-genes were described in acute or chronic hepatitis [178, 179] and in cells from hepatocellular carcinomas [180].

If mutations do not disturb the crucial parts of the transactivating domain of HBx, the HBV variant may remain replicative. One frame-shift mutation which was caused by an additional T within the X-C overlapping region was described [181]. The resulting HBx consisted of the first 151 aa of the wildtype virus and of 42 additional aa encoded by the C-ORF. This variant was shown to keep its transactivating function *in vitro* [182].

Mutants with a functionally inactive HBx are believed to restore their transactivation function by wildtype X-DNA which can be integrated into the host chromosomes. This concept was confirmed by the observation that a woodchuck carrying high levels of X-deletion-mutants in his serum had a normal WHV infection and X-DNA was detectable in his liver samples [175].

Additionally a HBV variant carrying a deletion which altered the region E of HBx could be isolated from the serum of an immunocompromised child who first was infected with the wildtype virus. The transcription of the mutated pregenomic RNA was observed to be normal and it may be supposed that the functional HBx was encoded by the integrated wildtype HBV [183].

Truncated versions of the X-gene frequently were identified to be integrated into the host chromosomes in patients with chronic hepatitis [18] or with hepatocellular carcinoma [180, 184, 185]. They therefore may be speculated to play a role in the genesis of the HBV associated liver cancer.

X-mutants might cause important problemes in blood donors. They are suspected to lead to a transmissible HBV infection which cannot be detected by conventional test systems because the classical serological markers HBsAg and HBeAg can be missing.

There are various explanations why the serum level of X-mutations was found to be inversely correlated with the serum viral load of the wildtype HBV [175] or with the appearance of HBsAg and HBeAg [175, 178, 179, 186].

First HBx is supposed to be important for the viral gene expression and thereby for the replication *in vivo* [175, 178, 179, 186, 187, 188]. Interestingly HBx does not seem to be essential for the viral life cycle *in vitro* [189, 190]. A low level of functionally HBx could result in a reduced expression of the S-gene or the C-gene. HBx-variants without a functional transactivating domain therefore could decrease the HBsAg or the HBeAg serum level. Furthermore many X-deletions were described to affect the precore-start-codon thus leading to a HBeAg-negative serotype [175, 186].

On the other hand the X-deletion-mutants may interfere with the replication and the packaging of the wildtype virus and thereby reduce the serum level of the wildtype HBV [175].

The strengh of the host immune response and thereby the severity of the liver disease was described to be influenced by the wildtype / X-

deletion-mutant ratio [186, 188]. If X-mutated viruses were predominant over the wild type virus in the infecting inocculum the HBV could be tolerated by the immune system because the S-gene and the C-gene were not expressed. It resulted a HBsAg-negative and HBeAg-negative hepatitis. In these cases antibodies against the viral polymerase were detectable which implies that the virus replicated at a very low level [188]. In the following time period the wildtype HBV became predominant and the patient´s immune system was stimulated. Now antibodies against HBx, antiHBs and antiHBe could be detected. Thus the immune system mostly eliminated the wildtype viruses and the X-deletion-mutants again became predominant.

From the described course of infection it might be concluded that anti-HBs is not protective against X-deletion-mutants. This might explain that HBV can chronically persist after the seroconversion to antiHBs-positive [186].

X-gene deletion mutants were described in Italy [175], in the USA [188], in Germany [183], in Egypt and in Japan [178].

The gene regulating elements within the X-region

The X-ORF also contains of the core-promoter/enhancer II complex. This regulatory element is composed by the core-promoter (NT 1586 to NT 1849) [191, 192] and the enhancer II (NT 1682 to NT 1800) [193, 194]. The sequence NT 1742 to NT 1849 is called the basic core promoter [195]. It is supposed to be sufficient for the correct initiation of the transcription of the pregenome and of the precore mRNA. The enhancer II was found to regulate the activity of the promoter of the S-gene [193, 196].

Point mutations within the core-promoter/enhancer II complexe were found in Asian patients with fulminant and chronic hepatitis (NT 1653 (C to T), NT 1752 (T to C), NT 1762 (A to T) and NT 1764 (G to A) [93, 197, 198, 176, 199, 200]), in patients from Israel with fulminant hepatitis (NT 1766 (C to T), NT 1768 (T to A) [93, 201]), and in immunocompromised patients (NT 1714 (A to G), NT 1718 (T to C), NT 1729 (A to G), NT 1746 (G to C) [36], and NT 1764 (G to T) [44]). They were more frequent in genotype C than in genotype B [202]. These mutations might change the binding affinity of nuclear proteins and thereby alter the transcriptional activity of the HBV-DNA [197]. *In vitro* assays showed that the mutations AGG to TGA at NT 1762-1764, GTC to TTG at NT 1764-1766 or CTT to TTA at NT 1764-1766 replicated at a higher level [203, 204, 205, 201] and had an increased HBcAg expression whereas the expression of HBeAg was reduced [206, 207, 44].

Interestingly the core-promoter mutation AGG to TGA at NT 1762-1764 was also found in asymptomatic carriers [208, 209], and the patients had a low virus load. HBeAg expression generally was not prevented by core-promoter mutations but by precore-mutations, especially by the precore-stop-mutation G to A at NT 1896 [92, 200, 153, 210]. HBV strains of genotype A however where the mutation NT 1896 G to A is disadvantageous for virus replication had a decreased HBe expression by core promotor mutations [211].

Deletions or insertions within the core-promoter/enhancer II complex also were described. They were localized between NT 1748 to NT 1776 and spanned different sizes [178, 187, 176]. These mutations affecting the basic core-promoter mostly disturbed the initiation of the transcription of the pregenome and of the precore mRNA. This could be proved *in vitro* [190]. Additionally the nucleotide sequence of the enhancer II could be changed and thereby the expression of the S-gene be disturbed [178]. This might result in a hepatitis without the classical serological markers.

However recently an 11-bp insertion within the core-promoter was found in an immuno-compromised patient with fulminant hepatitis. This insertion created a new binding site for hepatocyte nuclear factor (HNF) 1, and the mutated strain was highly replication competent [142].

The HBV genome possess two direct repeats of 11 bp which lie within the core-promoter sequence. They are called DR1 (NT 1824 to NT 1834) and DR2 (NT 1590 to NT 1600) [188].

The direct repeat sequences are considered to be important for the reverse transcription. The synthesis of the minus-strand seems to be initiated within the hairpin structure of the precore region. A short nucleotide sequence is synthesized within the hairpin structure, transferred to a 4-nucleotide sequence homology within DR1 and then used as a

primer for the minus-strand-synthesis [72]. After the minus-strand-synthesis is terminated the pregenomic RNA is nearly almost degraded except some nucleotides at the 5´terminus. This oligonucleotide then is transferred to the DR2 within the minus-strand where it serves as a primer for the synthesis of the plus-strand [ref. in 212].

Today one point mutation (T to C at NT 1590) within the DR2 could be observed in a patient [178]. In the duck modell HB viruses with deletions within the DR1 or the DR2 were still able to replicate [213]. It therefore was speculated that the human HBV variant also might replicate at a decreased level [178]. Unfortunately no experiments were carried out with the human HBV-mutant.

The part of the HBV-genome which spans between the DR1 and the DR2 is called the cohesive end region. This region is considered to be the preferred site where HBV-DNA is integrated into host chromosomes [214, 215, 216]. This might be explained by the fact that the cohesive end region near the DR1 was found to be a preferred cleavage site for the topoisomerase I. This enzyme was able to catalyse illegitimate recombinations between HBV-DNA and cellular-DNA [215, 216]. The event of DNA-integration was found to be associated with deletions [214, 215].

The P-gene

The P-ORF encodes for a multifunctional enzyme (831 aa; 93 kDa) which is called the polymerase (Pol). The polymerase is responsible for the reverse transcription of the pregenomic RNA to the double stranded DNA.

Four different domains within the P-gene product could be distinguished (Fig. 5) [217, 218, 219].

The terminal protein is considered to be the part of the polymerase where the synthesis of the minus-strand-DNA is initiated. First the terminal protein binds covalently a T. This T will be the 5´terminus of the minus-strand which has been mapped to NT 1827 [212]. Interestingly in experiments the human polymerase was able to accept all of the four nucleotides as the first base of the minus-strand. If the terminal protein covalently binds a G then the 5´terminus of the minus-strand may result to be G 1826. This is consistent with the observation that the 5´terminus of the minus-strand was mapped to T 1827 and to G 1826 [220]. However the strenght of the protein-nucleotide-interaction was found to be decreasing from T to G to A to C [212]. This order corresponds to the nucleotid sequence (A (NT 1827) C (NT 1826) U (NT 1825)) within the DR1. Once synthesized the whole minus-strand-DNA remains protein-bound with its 5´ terminus [221].

In the duck-HBV the first nucleotide of the minus-strand (dGMP) is bound to the OH-group of Tyr-96, a part of the terminal protein, by a phosphodiester [222]. Depending on the template also other nucleotides can be accepted by Tyr-96 [223]. Tyr-96 was described to be indispensible for the enzyme function. Furthermore a functional reverse transcriptase region also seems to be required for the priming activity of the duck-polymerase [222].

One point mutation in the terminal protein was detected in a patient with a latent HBV infection and a serological immunity. The changed polymerase molecule was unable to carry out the encapsidation of the pregenome and the virus

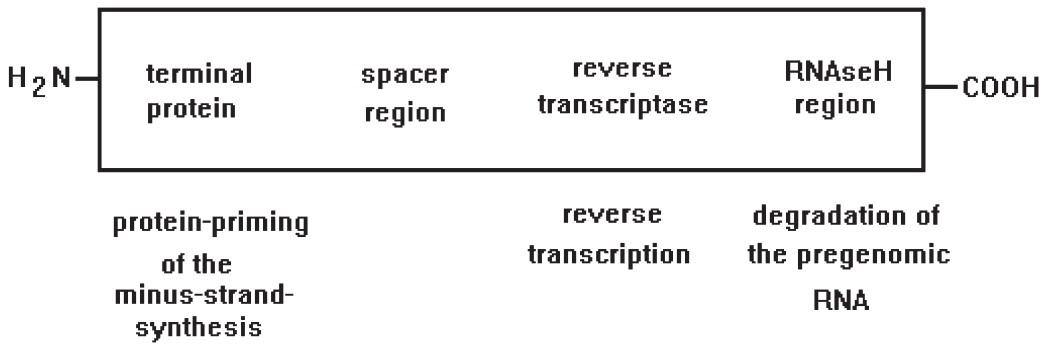

Fig. 5 Structure and functions of the polymerase.

replication was terminated. This defect could be restored by normal polymerase [224].

The spacer region was described to be dispensable for the enzyme function and easily tolerates mutations [218, 219].

The catalytic center of the reverse transcriptase was mapped around the aa-sequence YMDD (Tyr – Met – Asp – Asp). This region seems to be the nucleotide recognizing site of the enzyme [225]. The YMDD-motif was found to be highly conserved among retroviruses and hepadnaviruses [226]. In the human HBV genome YMDD (aa 551 to aa 554) is encoded by the NT 736 to NT 747.

Mutations of the YMDD-sequence were found to be of clinical interest.

The nucleoside analogue lamivudine [(-) ß-L-2', 3'-dideoxy-3'-thiacytidine)] actually is used in treatment of chronic Hepatitis B. Unfortunately HB viruses which changed the YMDD-motif to YVDD or to YIDD were found to be lamivudine-resistant [11, 226], and patients infected with YMDD mutants had the same clinical and pathological course as patients chronically infected with the wildtype virus [227].

It was observed *in vitro* that the YMDD-mutants replicated at a very low level. If these variants additionally to the YMDD-mutation had Met-528 instead of Leu they replicated at a higher level than viruses carrying only the YMDD-mutation [228]. Assays with transiently transfected cells revealed an increasing lamivudine resistance from wildtype strains to single mutated strains (L528M or M552V) to strains with the M552I mutation and to double mutated strains (L528M plus M552V or L528M plus M552I) [229, 77]. Therefore mutations within and outsite the YMDD-domain might be selected during lamivudine monotherapy and could contribute to therapy failure [230]. *In vivo* the Leu to Met mutation at aa 528 has been observed in association with the mutation YVDD [231] and in association with the mutation YIDD [232]. Natural occurring YMDD variants frequently exhibit other aa substitutions like the change Leu-568 to Val [43], Ser-561 to Thr [233], Ile-511 to Val, Ala- 548 to Val, Ser-567 to Ala, Ala-570 to Thr [234] or Val-521 to Leu [235]. The later mutation and the mutation Leu-528 to Met additionally to lamivudine resistance may cause resistance to the anti viral drug famciclovir [236, 237].

It might be speculated that lamivudine resistant HB strains already exist in a minority before treatment starts. The lamivudine monotherapy decreases the replication of the wildtype virus and the resistant mutants that replicate at a very low level become preponderant over the wildtype virus only after a long-term treatment [238, 239]. Interestingly the liver disease did not relapse in all patients where lamivudine resistant variants could be found after long-term treatment [238, 239]. After cessation of lamivudine treatment the wildtype virus that has remained in hepatocytes during therapy starts replicating at normal level and become preponderant over the mutant virus. Finally the drug resistant variants became undetectable [238] because their number of genome copies is below the detection limit.

After failure of lamivudine monotherapy additionally famciclovir was used in a study. Due to the new selection pressure by lamivudine and famciclovir together, preexisting mutants that were resistant to both drugs appeared and maintained the liver infection [232].

It also should be mentioned that some centers use the orthotopic liver transplantation together with lamivudine to treat the HBV-induced liver cirrhosis [226]. After successful liver transplantation and under lamivudine treatment HBV-DNA generally was no longer detectable in serum samples. However some patients developed a new HBV-viremic stage several months after transplantation in spite of the ungowing lamivudine treatment. This HBV reactivation could be associated with YMDD-mutants [234]. Interestingly this new HBV-viremia did not always lead to a hepatic decompensation or a fibrosing cholestatic hepatitis [11, 226, 240].

Point mutations which affect the YMDD-sequence of the polymerase also might change the aa-sequence encoded by the S-region because the S-ORF is entirely inclused in the P-ORF (see fig.1). However the concerned nucleotides (NT 736 to NT 747) are situated downstream of the nucleotides which encode for the antigenically relevant parts of the different alleles within the S-gene. Therefore YMDD-mutations of the polymerase may not be correllated with changes of the serotypes of the small-HBsAg [240].

Finally it is notable that single point mutations like Phe-501 to Leu or Leu-515 to Met [228] and the double point mutation Leu-528 to Met/Phe-514 to Leu [241] also were found in lamivudine-

treated patients without changes in the YMDD-motif. While the replication of the Met-515-mutant was hardly reduced compared to the wildtype virus, the Leu-501-variant replicated at a very low level. The decreased replication was supposed to be due to a reduced encapsidation of the pregenomic-RNA [228].

Another point mutation which also prevented the encapsidation of the pregenomic-RNA was detected within the RNAseH region of the duck-HBV [242].

The aa-sequences 455 to 463, 551 to 559, 575 to 583, 655 to 663, 773 to 782 and 816 to 824 are situated within the reverse transcriptase domain or the RNAseH domain of the polymerase. They were found to be highly conserved. CD_8^+ cytotoxic T-cells against these 6 epitopes were described in patients with acute hepatitis. It therefore might be speculated that these CTL contribute to the viral clearence or the development of subclinical diseases [130, 243].

The epitope aa 455 to 463 (GLSRYVARL) was observed to be able to induce cytotoxic T-cells. Mutations could change the aa-sequence to GLPRYVARL, GVSRYVARL, GLPRYVVCL or GVSRYVARL, respectively. The mutated peptides were no more recognized by wildtype specific cytotoxic T-cells. Furthermore it has been found that the sequence GLPRYVARL was unable to induce cytotoxic T-cells [243].

In addition it was speculated that Ser-457, Ala-461 or Arg-462 of the GLSRYVARL sequence are the contact sites for the TCR. If theses critical aa are changed by mutations the TCR binding activity might be reduced and the CTL-mediated immune response be attenuated [243].

Interestingly, American patients with chronic hepatitis were observed to have only a week CTL response [130, 243]. Additionally patients whose CTL could not be stimulated by HBV antigens *in vitro* were not infected with variant viruses [243]. These observations seem to confirm the concept reported in “The C-gene” that in American patients a weak immune system which allows a great number of hepatocytes to be infected and not immune escape mutants might be responsible for the development of chronic liver disease.

Finally, it is nothworthy to mention another mechanism of viral persistence which also might contribute to the development of chronic hepatitis. One case of an acute hepatitis B was described where the wildtype HBV-DNA remained detectable in trace amounts in the serum although the patient clinically and serologically seemed to be cured. It therefore was speculated that HBV could have persisted in cells which are not reached by the immune system. Thereafter it may have been seeded into the circulation resulting in an ongowing stimulation of the CTL [243].

Mutants within the P-gene were described in patients from the UK [244], from the USA [11, 243], from Canada [226] and from Asia [240].

Epidemiology

The increasing knowledge about HBV variants requires new methods for epidemiological studies and for the screening of blood donors. The classical serological markers of HBV infection HBsAg, antiHBs, antiHBc, HBeAg, antiHBe are detected with commercial kits. The lower limit of detection for antiHBs in these kits is 10 mIU/mL. AntiHBs levels above this limit are considered to be protective [245, 246, 247], and according to the European Consensus Group on Hepatitis Immunity these persons do not need booster vaccinations [248]. However epidemiological studies in high prevalence areas found antiHBs negative persons that were antiHBc positive. One of these studies examined Taiwanese children vaccinated at birth [249]. At the age of 5 years 17% of the children were antiHBs negative. The annual rate of benign breakthrough infections associated with antiHBc seroconversion among these children was 2% but no child became HBsAg positive. Another study found three cases of hepatocellular carcinoma in HBsAg negative, antiHBc positive Taiwanese persons [250].

Therefore new techniques have been developed to detect the wildtype virus and the mutants.

The geographically widely distributed point mutation within the a-determinant Gly-145 to Arg [251] that leads to HBsAg negativity in some commercially available tests [252] can be detected by PCR. Specially created primers [38] introduce cleavage sites into the PCR products of the mutant-DNA but not into the PCR products of the wildtype-

DNA. The sensitivity of this method can be increased when 2 successive PCR amplifications are used. The first PCR amplificates both the wildtype-DNA and the mutant-DNA. Thereafter the second PCR uses primers that only adhere to the mutant-DNA. After restriction with an adequate enzyme the PCR products of the mutant-DNA will be fragmented but not the PCR products of the wildtype-DNA. This method may detect a concentration of 0.2% of the mutant-DNA in a mixed DNA-population [253] and therefore could be applied in epidemiological studies and in the screening of blood donors.

Novel radioimmunoassays also have been developed to detect HBs mutants. Their common principle is the use of different monoclonal antibodies directed to different epitopes within the S-gene products. These tests therefore may have a good chance to find a wildtype epitope. One attempt was made with a monoclonal antibody directed to the first loop of the a-determinant [254]. This test was shown to recognize HBV variants with mutations in the second loop of the a-determinant. Unfortunately the wildtype genotype D subtype ayw_3 could not be detected [254]. Other radioimmunoassays which recognized epitopes within the preS1 region, within the preS2 region, and within the S-region together [255] also were found to be efficient for the detection of mutations within the a-determinant [252]. The use of immunoassays might be a rapid and cheap method for the screening of blood donors and for the monitoring of vaccinated persons.

Three recently developed techniques may facilitate epidemiological studies with the common precore-mutation G to A at NT 1896 [110]. One of these techniques uses marked oligonucleotide probes that specifically bind to the distal precore-region [256] of the wildtype virus or of the 1896 G to A mutation, respectively. DNA amplification by PCR before hybridization assay increases the sensitivity of this method.

The amplification refractory mutation system technique might be another successful approach [257] for the epidemiology of the 1896 G to A mutation. This method uses 2 different PCRs with a wildtype-specific primer or a mutant-specific primer, respectively.

The third method that already has been described in detail above, uses specially created primers that introduce cleavage sites into the PCR products of the 1896 G to A mutant but not into the PCR products of the wildtype-DNA [258].

The most common mutations within the P-gene (YMDD to YVDD or YIDD) might get increasing clinical importance since nucleoside analogues are used for the treatment of HBV infections. The rapid detection of these mutants could be useful for therapeutic decisions and for the monitoring of patients treated with antiviral drugs. Therefore a line probe assay has been developped [77]. This test uses nitrocellulose-bound specific nucleotide probes that are hybridised with PCR products from the patient.

Another technique uses marked oligonucleotide probes designed for the wildtype-DNA-sequence or for a mutant-DNA-sequence, respectively. The probes are hybridised with the PCR products from the patient and then the melting temperature of the DNA-hybrides is detected. YMDD mutant-DNA can be distinguished from wildtype-DNA by different melting characteristics. This assay is very rapid but with low sensitivity [259].

Perspectives

The new methods in molecular biology and immunology allow us to better understand the HBV-host interactions. These virus-host interactions can be altered by the appearance of mutated viruses. This might lead to a changed clinical course of infection. Due to the high mutation rate of the HBV-genome, HBV-variants get a great clinical, diagnostic, and therapeutic importance. Therefore further investigations are necessary to answer the remaining questions about the properties of mutated hepatitis B viruses. These questions are as follows:

How is the manifestation rate of infections with mutated hepatitis B viruses?

Are mutated hepatitis B viruses stable in the environment?

Are there any geographical differences in the prevalence of mutated hepatitis B viruses?

Does the appearance of mutated hepatitis B viruses change the clinical course of other diseases?

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