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Hepatitis B virus genetic variability and evolution

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Abstract

Hepatitis B virus has been evolving gradually over a long period of time, resulting in a large amount of genetic diversity, despite the constraints imposed by the complex genetic organization of the viral genome. This diversity is partly due to virus/host interactions and partly due to parallel evolution in geographically distinct areas. Recombination also appears to be an important element in HBV evolution. Also, human intervention in the form of mass vaccination and antiviral treatment will reduce the burden of HBV-related liver disease but may also be accelerating evolution of the virus.
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1. Introduction

The association between man and hepatitis B virus (HBV) is certainly very ancient. The Hepadnavirus family comprises representatives from non-human primates that are very similar to HBV, from rodents (woodchuck hepatitis B virus, or WHV, etc.) that share about 80% similarity to HBV (Galibert et al., 1982) and from birds (duck hepatitis B virus, or DHBV, etc.) that share only about 40% similarity to HBV (Mandart et al., 1984). If one supposes a common ancestor and separate evolution within the different lineages, then hepadnaviruses must have existed before the speciation between birds and mammals. This longevity has permitted the emergence of a great deal of diversity, not only between the hepadnaviruses of the different species but also among HBV isolates, despite the severe constraints imposed by the genomic organization (Mizokami et al., 1997), the genomic structure and the replication strategy of Hepadnaviruses (Ganem and Schneider, 2001). The genomes of hepadnaviruses are very small, and DHBV, with only 3021 bp, possesses the smallest genome of known animal DNA viruses. This has forced the virus to optimize the genomic organization.

There is much overlap of genes and every nucleotide participates in the coding of at least one viral protein. The regulatory and structural sequences necessary for viral transcription and replication are therefore automatically included within coding regions. This extensive overlap limits the diversity that the virus can tolerate. A mutation may have little effect on one viral protein but may have severe consequences on an overlapping gene or on regulatory and structural sequences. The structure of the genome is a direct reflection of the viral replication strategy. The genome exists in fact in two different forms (Ganem and Schneider, 2001). In the virions, the genome is a relaxed circular DNA molecule (RC-DNA) that is only partially double-stranded (Fig. 1). One strand is complete (the L or minus strand) and even has a short terminal redundancy with a protein, the viral polymerase, covalently attached to the 5'-end. The other strand (the S or plus strand) is incomplete, about two-thirds complete for HBV, almost complete for DHBV. The 5'-end is fixed and starts with a short oligoribonucleotide and the 3'-end is variable. The plus strand overlaps the 5' and 3' extremities of the minus strand, thereby assuring the circularity of RC-DNA. RC-DNA therefore contains all of the genetic information of the virus but is unsuitable as a replication template. After infection of a hepatocyte, plus strand DNA is completed, the oligoribonucleotide at the 5'-end of plus strand DNA and the terminal redundancy of minus strand DNA, along with the attached viral polymerase, are eliminated and the two strands are ligated. Importantly, these steps
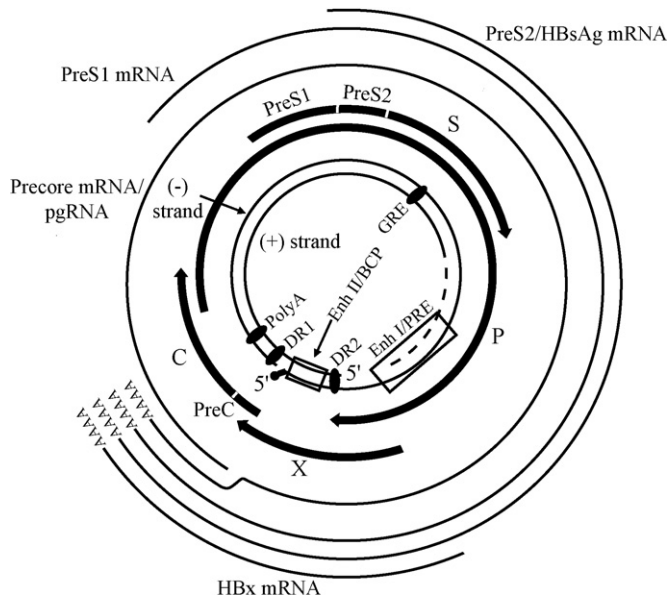
Fig. 1. Genetic organization of the HBV genome (RC-DNA form). GRE, glucocorticoid response element; Enh, enhancer; PRE, post-transcriptional regulatory element; BCP, basal core promoter; DR, direct repeat.

are apparently performed by cellular enzymes, independently of the viral polymerase. The genome is then found in the nucleus of the infected hepatocyte in the form of a covalently-closed circular DNA molecule (cccDNA) that is the real replication matrix. The mRNAs for the viral proteins are transcribed from the cccDNA. This includes pregenomic RNA (pgRNA) which is of more-than-genome length, approximately 1.1 genomes. In effect, transcription of pgRNA is initiated upstream of the unique viral polyadénylation signal. At the first passage of the polyadénylation signal, the nascent RNA is not, or only poorly, polyadenylated and efficient polyadenylation occurs only after the second passage. The pgRNA therefore possesses a terminal redundancy that is essential if the virus is not to lose genetic information during replication and can be compared to the LTRs of retroviruses. After export to the cytoplasm, the pgRNA is encapsidated along with the viral polymerase and minus strand DNA is synthesized via reverse transcription. The synthesis of plus strand DNA is initiated, but at some point there is maturation of the nucleocapsid containing RC-DNA and plus strand DNA synthesis stops. The nucleocapsid can then either be recycled back to the nuclease to amplify or replenish the cccDNA pool or it can be enveloped and secreted as a new virion. The duality of the genomic forms of hepadnaviruses has important consequences. The mutations that are generated by reverse transcription are initially found in RC-DNA. Mutations can be stably transmitted only if the mutated RC-DNA genome is recruited into the cccDNA pool of the patient, either by recycling to the nucleus or infection of a new hepatocyte by the mutant virus. In both cases, the mutant virus will be in competition with a vast excess of other viral genomes, either "wild type" or containing other mutations. The emergence of HBV mutants is therefore much slower, even in the presence of selective pressure, than with other viruses, such as HIV or HCV, where mutations generated by an error-prone replication step, reverse transcription or RNA-

dependant transcription, can have immediate phenotypic effects. A second consequence of the dual nature of the HBV genome is the presence in a chronically infected patient of two quasispecies. The first is the quasispecies of the cccDNA pool and the second is the quasispecies of RC-DNA that reflects both the cccDNA quasispecies and new mutations generated during replication. Finally, the diversity of HBV genomes can be divided into two categories, a genotypic variability that is the result of gradual evolution of the genome in the absence of selective pressure and phenotypic variability that results from adaptation of the virus to selective pressures, either the host immune response or antiviral treatments, including vaccination. With genotypic variation, viral fitness is the most important factor. A mutant virus that is significantly less fit than other circulating HBV will eventually be eliminated even if the mutated RC-DNA genome integrates the cccDNA pool. With phenotypic variation, the driving force is selection, since the ability to resist antiviral pressure usually far outweighs the lowered fitness of a mutant compared to a wild type virus that cannot resist.

2. Genotypic variation

2.1. HBV serotypes

Very quickly after the discovery of "Australia antigen", or HBsAg, the major envelope protein of HBV, it became evident that sera of patients who had seroconverted to anti-HBs did not react in the same way with HBsAg from different chronic carriers and that this was due to viral variability. The first classification of HBV isolates was therefore done by serotyping, the reactivity of the HBsAg of the isolate with standard panels of antisera (Bouvier and Williams, 1975; Courouce et al., 1983). The major immunogenic region, the "a" determinant spanning residues 124–147 of HBsAg and probably composed of several conformational epitopes, is common to the almost all HBV isolates and is therefore not informative for classification. Classification is therefore done using subtypes, the molecular basis of which are now known (Table 1). The two major subtype epitopes are the d/y and r/w determinants. Both of these determinants are comprised of two mutually exclusive epitopes that depend upon the nature of the amino acids at positions 122 and 160 of HBsAg respectively. If the amino acid at position 122 is Arg (122R)

Table 1
Molecular basis of HBV serotypes

Serotype	HBsAg sequence
ayw1	122R + 160K + 127P + (134F and/or 159A)
ayw2	122R + 160K + 127P
ayw3	122R + 160K + 127T
ayw4	122R + 160K + 127L
ayr	122R + 160R
adw2	122K + 160K + 127P
adw3	122K + 160K + 127T
adw4q−	122K + 160K + 127L + 178Q
adrq+	122K + 160K + 177V + 178P
adrq−	122K + 160R + 177A

then the subtype is *y*, and if it is Lys (122K) then the subtype is *d* (Okamoto et al., 1987). Similarly, 160R defines the *r* subtype and 160K defines the *w* subtype (Okamoto et al., 1987). The four possible combinations define the major subtypes and additional amino acids contribute to immunogenicity (Table 1), giving rise to 10 distinct subtypes (Norder et al., 1992a,b). While the ability to detect HBsAg was of obvious importance for the safety of the blood supply, serotyping was useful for epidemiological studies, including studies of nosocomial and iatrogenic infections and intra-familial transmission.

2.2. HBV genotypes

The first sequence of a complete HBV genome was published in 1979 (Galibert et al., 1979). By the end of the 1980s, enough full length genome sequences had accumulated in the databases to enable classification of HBV strains by genomic sequence rather than by surface protein antigenicity. Okamoto et al analyzed 18 full length genomes and divided them into four groups, or genotypes, named A to D (Okamoto et al., 1988). An HBV genotype was defined as a sequence or a group of sequences that diverges from known genotypes by 8% or more. Since then, four more human genotypes have been identified, E to H (Arauz-Ruiz et al., 2002; Naumann et al., 1993; Norder et al., 1994; Stuyver et al., 2000). There is a certain correlation between serotype and genotype, but it is far from perfect (Norder et al., 1992a; Ohba et al., 1995). For example, strains encoding an HBsAg of serotype *adw2* can be found in genotypes A, B, C and G (Table 2). The evolution of HBV is strikingly highlighted by the geographical distribution of the genotypes (Table 2). Genotypes A and D are ubiquitous, although genotype D is relatively rare in Northern Europe and the Americas. Little is known about the distribution of genotype G. It has been found in Europe, USA and Japan and may be ubiquitous. Genotypes B and C are found essentially in Asia. Genotype E is found in sub-Saharan Africa and rare cases in France and Britain may be due to immigration. Genotype F is found mainly in South and Central America and probably originated in Amerindian populations. Genotype H is found in Central America and in the southern part of the United States, and these latter may also be due to immigration. The significance of genotype F and H strains in Japan is not clear. However, genotype F has been described in Polynesia and at least one of the Japanese genotype F strains was isolated in Okinawa whose population is in part of Melanesian origin. Genotype F (and perhaps genotype H) may therefore be a Pacific/Latin America genotype. There is a great deal of diversity within the genotypes and this has lead to the division of some genotypes into subtypes (Table 2) (Devesa et al., 2004; Huy et al., 2004; Norder et al., 2004; Sugauchi et al., 2004a,b). Again, the origin of subtypes seems to be geographical, with different subtypes evolving independently of the others. Thus, subtype A1 (or Aa) is found mainly in Africa and Asia and subtype A2 (Ae) in Europe and North America (Sugauchi et al., 2004a) while B1 (Bj) is found in Japan and B2 (Ba) in the rest of Asia (Sugauchi et al., 2004b). Despite the subtypes and the frequency of strains with deletions or insertions, each genotype has a canonical size, ranging from 3182 nt for genotype D to 3248 for genotype G. Since the

coding capacity of the HBV genome is optimized, with overlapping open reading frames and each nucleotide participating in the encoding of at least one viral protein, these size differences affect one or more of the proteins (Table 2). For example, the P gene, coding the viral polymerase (Pol) partially overlaps the C gene that codes HBcAg. Genotype A strains have a two codon insertion near the end of the C gene and both HBcAg and Pol have two additional amino acids (aa). Conversely, genotype G strains have a 36 bp insertion immediately after the initiation codon of the C gene, increasing the size of HBcAg by 12 aa. This does not affect Pol, but a one codon deletion in the PreS1 region reduces both PreS1 and Pol by one aa.

Strains very similar to human HBV have been isolated from greater apes (Grethe et al., 2000; Lanford et al., 1998; Norder et al., 1996; Warren et al., 1999). They were first identified in captive animals and were thought to be human-to-ape transmissions. However, phylogenetic analysis clearly shows that they can be differentiated from the known human genotypes (Fig. 2). Most of the strains have been found in Old World apes, but the most divergent group has been isolated from a New World ape, the woolly monkey (Lanford et al., 1998). It is interesting to note that the New World human genotypes, F and H, lie on the same branch (Fig. 2), which may suggest that these human genotypes arose from zoonotic infections. As with Simian Immunodeficiency Virus, HBV infections in apes are generally asymptomatic, with few signs of liver disease, although the woolly monkey virus was originally isolated from an animal with fulminant hepatitis.

2.3. HBV genotypes and potential clinical outcomes

There have been several attempts to link a particular genotype to more severe liver disease, but the results are controversial. In countries where genotypes A and D co-exist, it has been suggested that genotype A infections lead to more chronic hepatitis than genotype D infections (Mayerat et al., 1999). However, in a report on long-term follow-up of chronic HBV carriers in Spain, spontaneous viral clearance was significantly higher in genotype A carriers than in carriers of other genotypes (Sanchez-Tapias et al., 2002). In several countries where genotypes B and C co-exist, genotype C infections seem to progress more rapidly to cirrhosis and hepatocellular carcinoma (HCC), which appears around the age of 30 years for those patients infected at birth (Ding et al., 2001; Kao et al., 2000; Sumi et al., 2003). However, by the age of 45 years, the incidence of HCC is the same for genotype C or genotype B infections (Sumi et al., 2003). The incidence of HCC may also be affected by factors other than HBV infection such as HCV co-infection, alcohol intake and aflatoxin B1 in the food supply. In the Amazonian basin, genotype F infections are associated with fulminant hepatitis, but this occurs in the context of co-infection or superinfection with hepatitis delta virus (HDV) genotype III (Casey et al., 1996; Quintero et al., 2001). The specific roles of HBV genotype F, HDV genotype III or a combination of the two in these fulminant cases is not known. In a cohort of HIV co-infected patients, infection with HBV of genotype G was strongly associated with increased liver fibrosis (Lacombe et al., 2006). Genotypes may also influence the outcome of treatment. In a trial of pegylated interferon (Janssen

Table 2
Characteristics of HBV genotypes and subtypes

Genotype	Subtype	Serotype[a]	Geographical distribution	Genome[b]	Pol[c]	HBcAg[c]	PreS1[c]
A	A1 (Aa)	**adw2**, *ayw1*	Africa, Asia	3221	845	185	400
	A2 (Ae)	**adw2**, *ayw1*	Northern Europe, North America				
B	B1 (Bj)	**adw2**	Japan	3215	843	183	400
	B2 (Ba)	**adw2**, *adw3*	Rest of Asia				
	B3	**adw2**, *ayw1*	Indonesia, China				
	B4	**ayw1**, *adw2*	Vietnam, Cambodia				
C	C1	**adrq+**, *ayr, adw2, ayw1*	Far East	3215	843	183	400
	C2	**adrq+**, *ayr*	Far East				
	C3	*adrq−, adrq+*	Pacific Islands				
D	D1	**ayw2**, *adw1, ayw1*	Europe, Middle East, Egypt, India, Asia	3182	832	183	389
	D2	**ayw3**, *ayw1*	Europe, Japan				
	D3	**ayw3**, *ayw2, ayw4*	Europe, Asia, South Africa, USA				
	D4	**ayw2**, *ayw3*	Australia, Japan, Papua New Guinea				
E		**ayw4**, *ayw2*	Sub-Saharan Africa, UK, France	3212	842	183	399
F	F Ia	**adw4**, *ayw4*	Central America	3215	843	183	400
	F Ib	**adw4**	Argentina, Japan, Venezuela, USA				
	F II	**adw4**	Brazil, Venezuela, Nicaragua				
	F III	**adw4**	Venezuela, Panama, Columbia				
	F IV	**adw4**	Argentina, Bolivia, France				
G		**adw2**	USA, Germany, Japan, France	3248	842	195	399
H		**adw4**	USA, Japan, Nicaragua	3215	843	183	400

[a] The main serotypes are shown in bold.

[b] Nucleotides.

[c] Amino acids. The sizes of HBsAg (226 amino acids), the PreS2 region (55 amino acids and HBxAg (154 amino acids) are identical for all genotypes.

et al., 2005), patients infected with genotypes A and B had a higher rate of HBeAg loss (about 45%) as compared to patients with genotype C or D (about 26%). On the other hand, in a Japanese study, while genotype B and C carriers responded well to interferon treatment, genotype A carriers responded poorly (Kobayashi et al., 2002). This discrepancy may be due to the type of interferon used, subtypes (probably Aa in the Japanese study and Ae in the European study), or to the small number of treated patients in the Japanese study. At present, HBV genotyping is not a standard procedure, but if more evidence accumulates that genotypes affect disease progression or treatment prognosis, then it may become so.
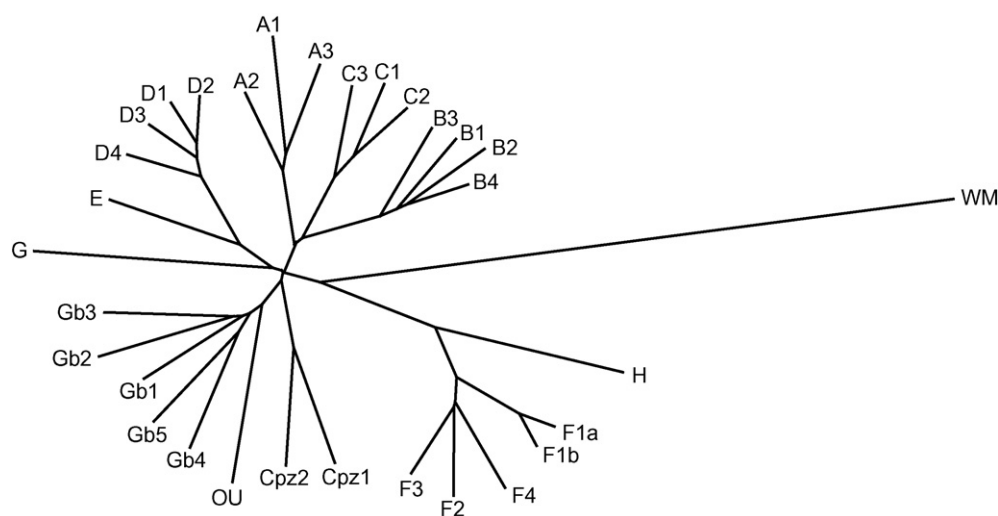

Fig. 2. Phylogenetic tree of HBV genotypes and subtypes. 884 full-length (>3100 nt) HBV genomes were aligned to identify genotype and subtype clusters. A representative sequence of each genotype or subtype was selected and re-aligned and the alignment was used to draw the tree. Accession numbers of the sequences used: A1, AB116092; A2, AY707087; A3, AB194951; B1, AB014366; B2, AY596111; B3, M54923; B4, AB073835; C1, AY123424; C2, AF223955; C3, X75665; D1, AB104712; D2, AB205126; D3, AY233291; D4, AB048702; E, AB205191; F1a, AY090459; F1b, AF223963; F2, AY311369; F3, AB036915; F4, AB166850; G, AB064312; H, AB059659; Cpz1 (chimpanzee), AY330911: Cpz2, AF242586; Gb1 (gibbon), AY330913; Gb2, Ay781181; Gb3, AY781186; Gb4, AJ131573; Gb5, AJ131574; OU (orang utan), AF193863; WM (woolly monkey), AF046996.

2.4. Recombinant genotypes

The classification of some isolates is difficult when the sequence is analyzed at the genome level. When analyzed at the gene level, some genes would cluster with the genes of one genotype while another gene would segregate with genes of a different genotype. The most striking example of intergenotype recombination is represented by the B2 (Ba) and B3 subtypes (and probably also B4). The strains in these subtypes are composed of a genotype B backbone but with a core gene and sometimes the core promoter being of genotype C origin. Only the B1 (Bj) subtype, found exclusively in Japan, seems to be "true" genotype B. Subtypes B2 and B3 are widespread in Asia outside of Japan and should probably be left within genotype B, but keeping in mind that they are recombinants and that this may affect their properties, pathological or otherwise. Recombinant genomes involving other combinations of genotypes have been described. Simmonds and Midgley have recently conducted a systematic search of complete HBV sequences in the databases for signs of recombination. They have found combinations of all the HBV genotypes except H. Recombination usually involves a single event, but more complex situations with apparently two or three events exist. There are also recombinations between human genotypes and primate sequences, including genotype C/gibbon recombinants, sequences that had previously been classified as subtype C4. This is surprising because the genomes were isolated from Australian Aborigines, and Australia is not a natural habitat for gibbons. It may reflect an ancestral recombination event that occurred during migration of the people that originally populated Australia.

The special structure of RC-DNA could lend itself to recombination events. However, HBV DNA replication occurs in isolation within capsids and it is thought that only one molecule of pregenomic RNA is encapsidated at a time. Also, it is hard to explain how the Ba isolates that contain not only the core gene of genotype C but also the core promoter could be generated during HBV replication. The chimerical genomes therefore probably arise by homologous recombination between cccDNA molecules. This would require the simultaneous infection of a hepatocyte with two different HBV strains and that the genomes of each strain are represented in the cccDNA pool. This is probably a rare event. However, such a scenario involving cccDNA molecules has the advantage that progeny recombinant virus can be immediately be produced by transcription of pregenomic RNA from the chimerical cccDNA molecule and this could explain why recombinant strains are so widespread. Intergenotype recombination is fairly easy to detect, but intragenotype recombination must also exist and may in fact be more important than intergenotype recombination. This not only contributes to the variability seen within HBV genotypes but also hampers our understanding of the evolutionary history of HBV.

3. Phenotypic variation

Phenotypic variants emerge in response to selective pressure. This pressure can be due to natural host immune-responses to the viral infection or to prophylactic or therapeutic measures (vaccination, antiviral treatments). The phenotypic variants are generally less fit than the normal genotypic variants. This is shown by the fact that they do not emerge as major viral populations in patients in the absence of selective pressure and, in the case of drug-resistant mutants, the drug-resistance mutations are usually rapidly lost when the drug is removed. For the virus, there is therefore a trade-off between decreased fitness and increased resistance, and this equilibrium will determine the ease with which a given mutant can emerge. In fact, much of the genotypic variation has probably been shaped by host immune responses, changes that result in little or no decline in viral fitness, any slight decline being compensated by a better adaptation to the host. Because much of the HBV genome consists of overlapping reading frames, the equilibrium is delicate. For instance, mutations that confer drug-resistance to the viral polymerase may provoke changes in the surface antigens, potentially affecting virion assembly, stability or infectivity (Zollner et al., 2001).

3.1. PreS1 and PreS2 mutants

Many of the mutations affecting the PreS domains of the envelope proteins are deletions. The region of the S gene coding the PreS1 and PreS2 domains is overlapped by the region of the P gene coding the spacer domain of the viral polymerase. This domain gives flexibility to the viral polymerase, but its sequence is not absolute and can suffer in-phase deletions and insertions without affecting the enzymatic activities of the protein. However, there are constraints on PreS1 mutations due to the fact that its N-terminus (residues 21–47 of the genotype D PreS1) is important in viral attachment to hepatocytes (Neurath et al., 1986) and the S promoter is located in the 3' extremity of the PreS1 coding region. In addition, some mutations can result in intracellular retention of the PreS1 protein (Bock et al., 1997; Melegari et al., 1994) which inhibits virion secretion and is cytotoxic. As a result, although PreS1 deletion mutants are replication competent, they usually need a helper virus and are found as minor viral populations. On the other hand, there appear to be few constraints on PreS2 mutations. The protein itself is present in variable quantities in different viral preparations and it is not essential for viral replication, viral particle morphogenesis and secretion and infectivity (Fernholz et al., 1993). Mutations include deletion or missense mutation of the PreS2 ATG, thereby abrogating synthesis of the protein, and deletions or alterations of B- and T-cell epitopes. PreS mutants emerge in chronic infections, often in patients treated with interferon (Gerner et al., 1998; Santantonio et al., 1992), and probably represent attempts by the virus to evade host immune responses. However, there is little evidence that PreS mutants are transmissible, and they therefore probably play a minimal role in HBV evolution, with one important exception. Compared to the other genotypes (Table 2), genotype D and non-human primate isolates have a 33 nucleotide deletion (or the other genotypes have an insertion) at or near the beginning of the PreS1 open reading frame. Nevertheless, a truncated but viable PreS1 protein, with a myristylation site and intact hepatocyte-recognition site, can be synthesized.

3.2. Core, precore and basal core promoter mutants

Many naturally occurring mutants of core protein (HBcAg) have been described (Akarca and Lok, 1995; Alexopoulou et al., 1997; Fiordalisi et al., 1994; Gunther et al., 1998; Ni et al., 2000; Okumura et al., 1996; Yuan et al., 1998; Zoulim et al., 1996). As with PreS mutants, these seem to represent desperate attempts to escape from host surveillance. Some are defective and require helper virus while others are stable, but it is not clear whether such mutants can be transmitted. Again, there are notable exceptions in two genotypes, A and G, that have 2-codon and 12-codon insertions in the C gene (Table 2). What can effectively be transmitted are mutants that affect the expression of precore protein. Both core and precore protein are coded by the C reading frame. Synthesis of precore protein is initiated from the first ATG of the frame and core protein from the second. The precore protein therefore initially contains all of the core protein sequence plus 29 amino acids at its N-terminus. These constitute a signal peptide that directs the nascent chain to the endoplasmic reticulum and the secretion pathway. The first 19 amino acids are cleaved off and during transport to the cell surface the protein is further matured by removal of the highly basic C-terminal tail. The protein is eventually secreted as a soluble antigen, HBeAg. The role of HBeAg is not fully understood (Milich and Liang, 2003). It is not essential for viral replication, secretion or infectivity and probably serves as a decoy, protecting infected cells from immune attack. Its major role would therefore be in the initial establishment of the HBV infection. However, the protein is very immunogenic and the host often mounts a strong anti-HBe response resulting in a crisis with elevated liver enzyme levels followed by decreased viral replication, although sometimes HBeAg can be lost without seroconversion to anti-HBe. For some time, it was thought that anti-HBe seroconversion was a marker of the end of active viral replication and resolution of the hepatitis, and it still remains an important end-point in antiviral therapy of HBeAg+ patients. However, it was shown in Greece that anti-HBe+ patients could still have active viral replication and liver disease (Hadziyannis et al., 1983). Such cases are now known to be a worldwide problem and are due to mutants that regulate the expression of HBeAg. Although replication is active, viral loads are generally several logs lower in HBeAg− patients than in HBeAg+ patients (Kessler et al., 2000). Also, children born to HBeAg+ mothers have a much higher risk of contracting a chronic HBV infection than children born to HBeAg− mothers (Stevens et al., 1979), consistent with the idea that HBeAg plays a role in the establishment of persistent infection, although viral loads may also be a factor. There are two classes of mutants that affect HBeAg expression, basal core promoter (BCP) mutants and precore mutants. The core promoter, positively and negatively regulated by Enhancer II and to some extent by Enhancer I, controls the transcription of precore mRNA and pregenomic RNA that can be the mRNA for both core protein and the viral polymerase and is the template for viral replication. It has been suggested that the two RNAs are controlled by two overlapping but distinct promoters (Chen et al., 1995). In strains isolated from anti-HBe+/HBeAg− patients, a double mutation is often found in the BCP, A1762T and G1764A

(Okamoto et al., 1994). There is much evidence that this double mutation is responsible for decreased precore mRNA synthesis (Buckwold et al., 1997; Li et al., 1999; Moriyama et al., 1996; Scaglioni et al., 1997). Li et al. have proposed that the double mutation eliminates a nuclear receptor binding site but in a favorable context it also creates an HNF1 transcription factor binding site (Li et al., 1999) and if this occurs then precore mRNA synthesis is inhibited but pregenomic RNA synthesis is unaltered or even enhanced. However, the double mutation also provokes two amino acid changes in HBxAg and altered transcriptional transactivation of the viral enhancers may also play a role in the specific inhibition of precore mRNA expression or enhanced replication. Given that HBeAg− patients generally have low viral loads, it is surprising that BCP mutants show enhanced replication in cell culture (Baumert et al., 1996; Moriyama et al., 1996; Parekh et al., 2003). In vivo and in the presence of reduced amounts of HBeAg the elimination of infected cells is probably more efficient and high replication in the surviving cells can ensure viral persistence. The double mutation itself leads to a twofold increase in viral replication, but when they are accompanied by mutations at positions 1753, 1766 and 1768, replication can be increased more than eightfold with respect to a genome without BCP mutations (Parekh et al., 2003). However, out-of-control replication allied with increased immune response can be dangerous, and BCP mutants have often been implicated in fulminant hepatitis and severe liver disease (Baumert et al., 1996; Friedt et al., 1999; Gunther et al., 1996; Sato et al., 1995). The second class of mutants affecting HBeAg expression, precore mutants, completely abolishes HBeAg expression by disruption of the precore reading frame. This is sometimes accomplished by missense mutation of the initiation codon or frameshift mutations of the precore region (Fiordalisi et al., 1990; Okamoto et al., 1990; Raimondo et al., 1990). However, by far the most prevalent precore mutant contains a nonsense mutation of codon 28 of the precore region, changing the codon from TGG (Trp) to TAG (Carman et al., 1989; Okamoto et al., 1990; Tong et al., 1990), sometimes accompanied by missense mutation of codon 29 from GGC to GAC. Codon 28 precore mutants are rare in genotypes A, F and H (Table 3), and this has been explained on a structural basis (Tong et al., 1992). The HBV encapsidation signal, essential for pregenomic RNA encapsidation and viral replication, overlaps almost all of the precore region and the beginning of the core protein reading frame. In the RNA, the signal forms a double stem-loop structure and nucleotide 1895, the nucleotide that is mutated from G to A to create the codon 28 mutant, is base-paired with nucleotide 1857 in the lower stem. In most HBV isolates, nucleotide 1857 is a T that can base pair with either 1895G or 1895A. However, in genotypes A, F, H and in some genotype C variants (Alestig et al., 2001) nucleotide 1857 is a C that can base-pair with 1895G in the wild-type sequence but not with 1895A in the codon 28 mutant. This would destabilize the encapsidation signal and lead to reduced replication and this is apparently sufficient to counter-select for codon 28 mutants in C1857 strains. This hypothesis has been confirmed by mutational analysis of the base-pairing requirements of the encapsidation signal (Tong et al., 1993) and by the fact that the rare genotype A isolates with a codon 28 mutation

Table 3
Frequency of basal core promoter (BCP) and precore mutants

Genotype/subtype	None	BCP	Codon 28	Both
A	80 (56%)	54 (38%)	1 (1%)	7 (5%)
B	61 (43%)	13 (9%)	47 (33%)	21 (15%)
B1	10 (31%)	1 (3%)	18 (56%)	3 (9%)
B2	42 (47%)	10 (11%)	23 (25%)	17 (18%)
B3	4 (67%)	0	2 (33%)	0
B4	5 (42%)	2 (17%)	4 (33%)	1 (8%)
C	77 (30%)	125 (48%)	13 (5%)	46 (18%)
C1	51 (28%)	78 (42%)	11 (6%)	44 (24%)
C2	23 (31%)	47 (64%)	2 (3%)	2 (3%)
C3	3 (100%)	0	0	0
D	62 (41%)	23 (15%)	41 (27%)	25 (17%)
E	29 (78%)	4 (11%)	0	4 (11%)
F	34 (83%)	2 (5%)	1 (2%)	4 (10%)
G	0 (0%)	0 (0%)	0 (0%)	13 (100%)
H	7 (70%)	3 (30%)	0 (0%)	0 (0%)
Primates	41 (89%)	0 (0%)	5 (11%)	0 (0%)

also have a compensatory mutation of nucleotide 1857 from C to T. On the other hand, isolates that normally have T1857 will form a wobble T–G base-pair in the wild type sequence and a stronger Watson–Crick T–A base-pair in the codon 28 mutant, stabilizing the encapsidation signal and potentially stimulating viral replication. This is apparently the case, with reports of enhanced replication of codon 28 mutants and an association with fulminant hepatitis (Hasegawa et al., 1994; Liang et al., 1991; Sato et al., 1995). However, the choice of the mechanism that a given genotype uses to regulate HBeAg expression is not fully explained by the structure of the encapsidation signal. In genotype C isolates there is a strong bias towards using BCP mutations (Table 3) that is not entirely due to the C1857 variants that are found in subtype C2. Conversely, genotype B isolates tend to acquire codon 28 mutations, even the B2 and B3 subtypes whose core promoters and precore/core genes are derived from genotype C, indicating that sequences outside of this region may influence the mechanism of choice. These differences between the two genotypes may be due to the fact that patients infected with genotype C take longer to seroconvert to anti-HBe than do patients with genotype B (Sumi et al., 2003). Many isolates have both BCP and codon 28 mutations. This probably occurs gradually, with the virus first reducing HBeAg expression through BCP mutations and then, with exacerbated immune pressure, abolishing entirely HBeAg expression with precore mutations. Consistent with this is the observation that BCP mutations are detectable in the late HBeAg phase of infection, whereas precore mutations emerge during anti-HBe seroconversion (Parekh et al., 2003). Interestingly, very few genotype E, F or H isolates have mutations, either BCP or codon 28. This is also the case for primate strains. Primate isolates are also different because in the majority of cases the wild-type codon 28 is TTG and not TGG, but they retain T1857. The encapsidation signal is therefore naturally weakened. This may be a virus/host adaptation and with less active viral replication there may be less selective pressure on the virus to develop mechanisms of regulating HBeAg expression. In fact, a codon 28 TAG mutation would seriously

strengthen the encapsidation signal and increased viral replication may be detrimental to the virus/host equilibrium. Finally genotype G has stably integrated both BPC and precore mutations into its genomes (Table 3). In spite of this, some genotype G infections have been found in HBeAg+ patients, and it has been suggested that the additional 12 amino acids at the N-terminus of HBcAg may confer "HBeAg-like" seroreactivity (Vieth et al., 2002). However, most, and perhaps all, genotype G infections reported so far seem to be co-infections with HBV genotype A. In a recent study that has well characterized acute genotype G mono-infections, the patients were HBeAg−/anti-HBe− (Chudy et al., 2006). It is possible that genotype G strains can only establish a persistent infection in association with a helper virus that can supply HBeAg.

3.3. HBsAg "vaccine escape" mutants

HBsAg is the main target for viral neutralization, either by natural or vaccine-induced anti-HBs. However, structural and genetic constraints limit major alterations of the protein. The complex secondary and tertiary structure of HBsAg is not yet fully understood. The basic working model is that of a protein with four transmembrane helices and with several residues at the N- and C-termini and a central major hydrophilic region (MHR, approximately residues 103–173) exposed at the surface of viral particles (Persson and Argos, 1994). The MHR itself is structured into five regions, including three central loops held together by disulphide bonds. The immunodominant "a" determinant (residues 124–147), against which most neutralizing antibodies are directed and which is the major target of HBsAg detection tests, is formed by loops 2 and 3. In addition, the region coding HBsAg also codes part of the reverse transcriptase/DNA polymerase domain of the viral polymerase. Some deletion mutants have been reported (Grethe et al., 1998; Takahashi et al., 1998), but they were isolated from long-term HBV carriers and may represent dead-end products. Small insertions in loop 1 of HBsAg have also been reported (Carman et al., 1995; Hou et al., 1995; Yamamoto et al., 1994). They were isolated from patients serologically negative for HBsAg, at least when monoclonal antibody based tests were used (Carman et al., 1995), indicating that although these insertions do not occur within the "a" determinant itself affect its structure. These insertion mutants presumably arose in response to immune pressure, but at least two patients were apparently anti-HBs negative (Hou et al., 1995) raising the possibility that escape from T-cell responses may also be a driving force in the emergence of HBsAg mutants. However, such insertion mutants are rare and the most common HBsAg immune escape mutants are due to missense mutations, often involving only one residue. These have been called "vaccine escape" mutants, but they can in fact be found in non-vaccinated people (Oon and Chen, 1998) where they exist as minor viral populations. They only emerge to become the major viral population in patients in the face of immune pressure, usually vaccine-induced or prophylactic treatment of liver transplant patients with human anti-HBs immunoglobulins (HBIg). The first vaccine escape mutant described (Carman et al., 1990) was substitution of

a Gly residue at position 145 by an Arg residue (G145R). Many other substitutions in the "*a*" determinant (I/T126A/N, A128V, Q129H/R, G130N, M133L/T, K141E, D144A/H) have since been associated with immune escape (Cooreman et al., 2001), but G145R is by far the most common variant. Immune escape mutants with substitutions outside of the "*a*" determinant have also been described (Oon et al., 1999), the most important of which is P120S/T. Many of the variant HBsAgs have been shown to have reduced affinity for monoclonal antibodies directed against the "*a*" determinant (Cooreman et al., 1999; Torresi et al., 2002) or to be less reactive in commercial HBsAg detection assays (Ireland et al., 2000), confirming that the substitutions are probably involved in immune escape. Oon et al have reported that a F183C variant isolated in Singapore also has reduced affinity for a monoclonal antibody (Oon et al., 1999). This is surprising because residue 183 is not just outside of the "*a*" determinant, it is not thought to be even in the MHR. Also, C183 is the normal residue for genotypes F and H, and this may mean that current vaccines may be less effective in countries endemic for these genotypes. G145R is by far the most common immune escape mutant, and the most studied. Several immune escape variants have been shown to be replication competent, stable over time, at least while anti-HBs selective pressure is present, and they can be transmitted (Ghany et al., 1998; Hsu et al., 1997; Okamoto et al., 1992; Protzer-Knolle et al., 1998). Most transmissions seem to be pseudo-vertical mother-to-child neonatal transmissions or host-to-graft transmissions in transplant patients, both types in the context of HBIg administration and vaccination, but there is some evidence of horizontal transmission of the G145R variant (Oon et al., 2000; Thakur et al., 2005). It has been reported that the G145R variant is defective in the secretion of infectious viral particles (Kalinina et al., 2003). Other immune escape variants may also have subtle defects in the virus life cycle that can explain why they do not constitute major viral populations in the absence of selective pressure and may limit horizontal transmission. The corollary of this is that mass-vaccination programs, although highly successful, will favorize the emergence of immune escape mutants to the detriment of non-mutated genomes. This seems to be the case in Taiwan where mass-vaccination started in 1984. At that time, HBV DNA prevalence in children was 8.6% and of these 7.8% had mutations in the MHR. Ten years after the start of vaccination, HBV DNA prevalence had fallen to 2.1% but the proportion of cases with MHR mutations was 28.1%, followed by a stabilization in 1999 with less than 1% DNA positive cases and 23.1% with MHR mutations (Hsu et al., 2004). HBsAg immune escape mutants will therefore play a role in the modern evolution of HBV. However, mathematical modeling predicts that even with worst-case scenarios it will take over 50 years before mutants such as G145R becomes predominant (Wilson et al., 1999). In fact, one of the mysteries about HBsAg vaccine escape mutants is why they are not more common. Almost all require only a point mutation and, given the error-prone HBV replication pathway, genomes with potential immune escape mutations must be generated frequently. There are several non-exclusive reasons that can explain this situation. First, initial inoculations may be small and may be eliminated before immune escape mutants can be generated. Secondly, before being stably propagated the mutant genomes have to integrate the cccDNA pool and any deficiency due to the mutations may have an important influence on this probability. Third, host anti-HBs responses may be directed not just against epitopes that are affected by the particular mutation. This would mean that HBsAg immune escape mutations are essential but not sufficient and can only emerge in patients whose anti-HBs responses are basically limited.

3.4. Polymerase drug-resistance mutants

The selection of drug resistant mutants is dependent on several cellular and viral factors. During chronic HBV infection, infected hepatocytes have usually a long half-life because of the defective antiviral immune response. Furthermore, these cells harbor viral cccDNA which seems to have a rather long half-life, at least in non dividing cells. It was shown that during antiviral therapy the decline in cccDNA levels in the liver is slower than that of total intrahepatic viral DNA and of serum viral load (Sung et al., 2005; Werle-Lapostolle et al., 2004). Furthermore, it parallels the decrease in serum HBsAg which may represent a surrogate non-invasive marker of intrahepatic cccDNA (Werle-Lapostolle et al., 2004). Because of the defect in the CD4 and CD8 T-cell response against HBV epitopes, this leads to the persistence of viral genome replication in the infected liver. Because of the spontaneous heterogeneity of the viral genome, this leads to the generation of complex quasi-species which composition evolves over time during the natural history of infection depending on the selective pressures. During antiviral therapy with nucleoside analogs, HBV mutants harboring mutations in the viral polymerase gene that confer resistance to antiviral drugs may then be selected. The emergence of drug-resistant mutants occurs in several phases (Fig. 3). At the start of treatment, there is a rapid fall, usually biphasic, in serum HBV DNA levels, often to levels undetectable by conventional assays. This is accompanied by a return of serum transferases to normal titers. However, in the absence of seroconversion, especially to anti-HBe, viral replication persists. During this phase, genotypic resistance can occur, i.e. although there are no serological signs of viral resistance, when viral sequences are amplified using sensitive assays, one can find a mixture, in variable proportions, of wild-type and mutant genomes. It can take several months after the initial detection of drug-resistant mutants before serum HBV DNA loads rebound, followed by an increase in serum transferase titers (phenotypic resistance). At this point, virtually all viral genomes contain the drug-resistance mutations. The rapidity of the selection process depends on the fitness of the specific mutants and the replication space available for the mutant to spread in the infected liver (Pult et al., 2001; Zhou et al., 1999). In turn, this leads to the rise in viral load which may result in treatment failure depending on the vigor of the immune response and the pharmacokinetics characteristics of the drug (host concentration of the drug versus the IC50 of the drug against the resistant mutants). The main polymerase gene mutations conferring resistance to nucleoside analogs have been characterized by in vitro phenotypic studies (Fig. 4). As the tri-dimensional structure of the viral polymerase
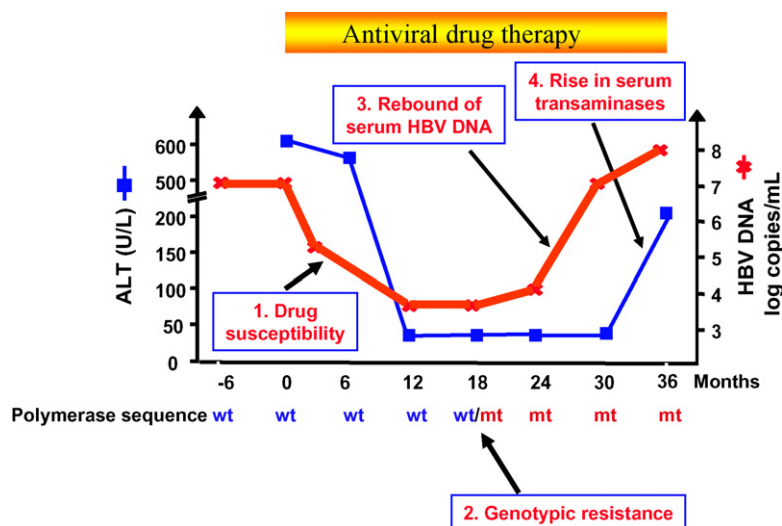
Fig. 3. Serological signs of emergence of a drug-resistant mutant.

is not yet known, the molecular mechanism of drug resistance for each amino acid substitution in the viral enzyme has been modelized by homology with the HIV reverse transcriptase for which a detailed knowledge of its structure and the implication of mutations for drug resistance are known (Allen et al., 1998; Bartholomeusz et al., 2004; Das et al., 2001). Lamivudine resistance mutants harbor a M204V or I substitution in the YMDD motif of the C domain of the reverse transcriptase domain. It has been hypothesized that these mutations affect lamivudine triphosphate efficacy by a mechanism of steric hindrance in the catalytic site of the viral enzyme (Allen et al.,

1998). As these substitutions affect the enzymatic activity and the replication capacity, compensatory mutations restoring some levels of replication capacity have been described, i.e. V173L and L180M (Delaney et al., 2003). Adefovir resistance mutants harbor a N236T and/or A181V amino acid substitution in the D and B domains of the viral polymerase respectively (Angus et al., 2003; Villeneuve et al., 2003). A A181T substitution has been also described but its biological and clinical significance remains to be determined. Several cases of entecavir resistance have been characterized. Entecavir resistance mutations occurred on a background of lamivudine resistance, as these patients received

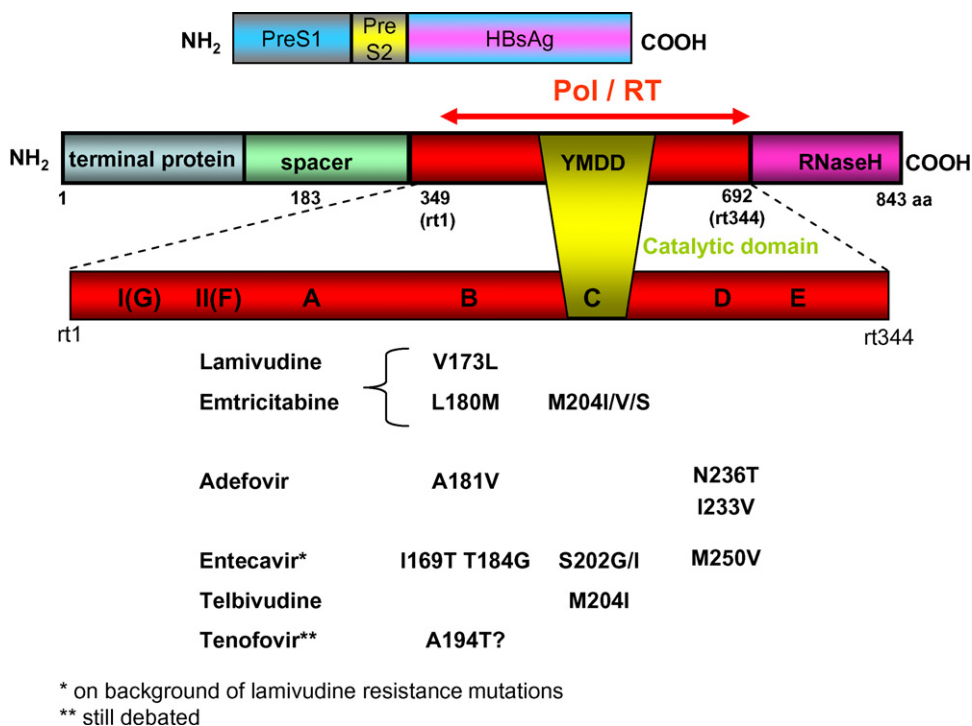* on background of lamivudine resistance mutations
** still debated

Fig. 4. HBV polymerase drug-resistant mutants. Schematic representation of the HBV polymerase and the envelope proteins whose open reading frame overlaps the polymerase gene. The four domains of the polymerase are indicated and the Pol/RT domain has been enlarged to show the well conserved sub-domains. The numbering system used to identify drug-resistant mutants follows the recommendations of Stuyver et al. (2001).

entecavir for lamivudine failure, with a combination of substitutions I169T and M250V, or T184G and S202I. These additional mutations clearly conferred an increased level of entecavir resistance compared to the initial lamivudine resistant strain (Tenney et al., 2004). Resistance to telbivudine has been observed in phase II trials in 4.5% of patients, and associated with a M204I mutation in the viral polymerase (Lai et al., 2005). Two cases of resistance to tenofovir have been identified in HIV-HBV co-infected patients receiving lamivudine and tenofovir; a A194T mutation was found in addition to lamivudine resistance mutations in these patients (Sheldon et al., 2005), but this was not confirmed by another study (Delaney et al., 2006). It should also be kept in mind that many polymerase gene mutations may also result in mutations in the overlapping surface gene. The combination of polymerase and surface gene mutations may then result in viruses that exhibit a reduced fitness which may translate for instance into differences in selection kinetics. Zollner et al. have reported that patients with HBV of serotype *ayw* (mainly genotypes D and E) have a 20-fold less risk of developing resistance to lamivudine than patients with serotype *adw* and that this seems to be due alterations in HBsAg introduced by the lamivudine-resistance mutations (Zollner et al., 2001). However, very few studies have been performed *in vitro* to gain insight into the infectivity of the drug resistant mutants. Such studies are hampered by the difficulty of working with primary human hepatocytes, the only cellular system that is available for these investigations. Bartholomew et al have assessed the susceptibility of HBV to lamivudine by infecting primary human hepatocytes with serum samples taken before the start of treatment and after viral breakthrough in varying concentrations of the drug (Bartholomew et al., 1997). The results demonstrated that the viral strains identified at the time of viral breakthrough were indeed resistant to lamivudine but also that these isolates were infectious for hepatocytes, leading to the possibility that these mutants may be transmissible (Thibault et al., 2002). However, this report involved a case of primary transmission of a lamivudine-resistant mutant to an HIV-positive patient who was receiving lamivudine. From the evolutionary point of view, the crucial question is whether a drug-resistant mutant is stable when transmitted to a naïve subject who is not being treated with the drug. We know that when a lamivudine-resistant mutant emerges and lamivudine treatment is stopped, then wild type virus rapidly replaces the mutant. However, we do not know if this occurs through true reversion, i.e. when RC-DNA is produced from mutant pgRNA, polymerase errors re-introduce the wild type sequence and this wild-type virus outgrows the mutant. In this case, the drug-resistant mutants are inherently unstable and will only persist as major viral populations in individuals treated with the drug. On the other hand, it is possible that the reappearance of wild type virus as the major population after cessation of drug treatment is due to persistence of non-mutated cccDNA molecules even after long-term drug treatment. In this case, drug resistant mutants transmitted to a naïve subject may be stable since there will be no competition with wild type virus. This can probably not be tested in the primary human hepatocyte system which is too short-lived. It may be possible to test the hypothesis in the trimeric mouse model (Ilan et al., 1999) but the answer will probably come from the clinic. If drug-resistance mutations are stable upon transmission to naïve individuals, then they will eventually become a permanent part of the HBV genetic landscape.

4. Conclusions

HBV has been evolving gradually over a long period of time, resulting in a large amount of genetic diversity. This is partly due to virus/host interactions and partly due to parallel evolution in geographically distinct areas. Recombination also appears to be an important element in HBV evolution. With increased migration and adoption of children from countries endemic for HBV and with mass travel (including sexual tourism) to these countries, the geographical distinctions will become more blurred. Also, human intervention in the form of mass vaccination and antiviral treatment will reduce the burden of HBV-related liver disease but may also be accelerating evolution of the virus.

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