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REVIEW ARTICLE

Genetic variability in hepatitis B viruses

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In 1988, it was reported that the full nucleotide sequences of 18 hepatitis B virus (HBV) strains clustered into four genetic groups (A to D) with more than 8% divergence between the groups. This classification of strains in terms of genome sequence has since proven to be an important tool in the understanding of HBV epidemiology and evolution and has been expanded to include three more genotypes. In parallel with the HBV genotypes described in humans, HBV strains isolated from different primates and hepadnaviruses found in woodchucks, ground squirrels, ducks and herons have been studied. Sequence differences between HBV genotypes can lead to structural differences at the level of the pregenome and can also lead to dramatic differences at the translational level when specific and commonly occurring mutations occur. There is increasing evidence that the clinical picture, the response to treatment and the long-term prognosis may differ depending on which genotype has infected the patient. The consideration of traditional serological patterns in a patient must therefore take the genotype of the infecting strain into account. Nucleotide variability between HBV strains has been used in several studies to trace routes of transmission and, since it is becoming increasingly clear that the differences between HBV genotypes are important, the need for reliable and easy methods of differentiating HBV genotypes has arisen. This review summarizes the knowledge of HBV genotypes with regard to their genetic, structural and clinically significant differences and their origin and evolution in the context of the hepadnaviruses in general.

Introduction

When Blumberg *et al.* (1965) first reported their findings on the 'Australia antigen', later to be known as the surface antigen (HBsAg) of hepatitis B virus (HBV), they included some epidemiological results that continue to be valid today. Thus, a higher prevalence of Australia antigen was found in normal populations from Taiwan, the eastern Mediterranean region, some Polynesian islands and Australian aborigines, than in North America. By establishing a correlation between acute or chronic hepatitis and the presence of Australia antigen in serum, the agent of serum or inoculation hepatitis was found. When serum from Australia antigen-positive patients was studied by electron microscopy, vast numbers of spheres and filaments of 22 nm in diameter were seen, but also larger particles of 42 nm with a central nucleocapsid and an outer coat (Dane *et al.*, 1970). These Dane particles were subsequently shown to constitute the complete virion and the smaller filaments and spheres were found to be excess Australia antigen or HBsAg. HBV has since been characterized into different antigenic subtypes and later into nucleotide divergence-based genotypes. Genotypic variation amongst HBV strains and how this genetic heterogeneity relates to HBV antigenicity and many other facets of HBV infection are the subject of this review.

Before the advent of PCR, the molecular characterization of HBV was a cumbersome process, as it was shown very early on that HBV would not grow in cell culture and was highly species specific, only infecting humans and some other primates. HBV was the first member to be discovered of a family of viruses, later designated *Hepadnaviridae*. This family has since been divided into two groups, the orthohepadnaviruses and the avian hepadnaviruses. These are hepatotropic, partially double-stranded DNA viruses. Their replication strategy is unique for animal DNA viruses and only shared by

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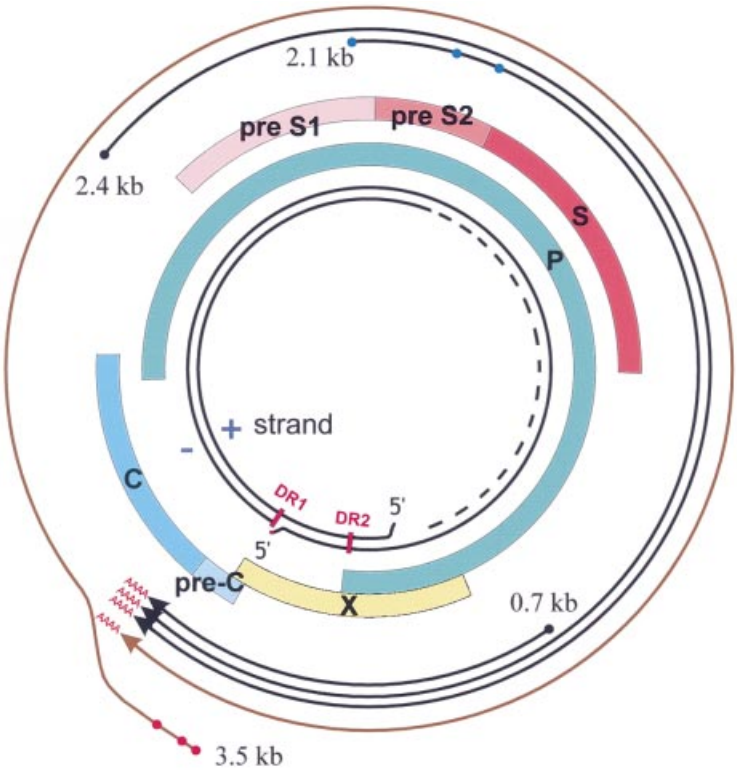

Fig. 1. Schematic representation of the HBV genome showing four main classes of transcript (arrows), the longest of which corresponds to pregenomic RNA (brown). Variations in 5′ end positions for the 2·1 and 3·5 kb size classes are shown with blue and red dots, respectively. Coloured boxes represent protein-coding regions. DR1 and DR2 are 11 bp repeat sequences with template functions during replication.

cauliflower mosaic virus (also a DNA virus), in that they use an RNA intermediate and a reverse transcription step (Seeger, 1991). The circular genome is very compact, with four partially overlapping open reading frames (ORFs) (Fig. 1). There are no non-coding regions in the genome, so that all regulatory signals are also part of protein-encoding sequences; HBV can encode approximately 50% more protein than would be expected from its genome length (Ganem & Varmus, 1987). In terms of HBV evolution, this leads to two opposing tendencies: the use of reverse transcriptase with its lack of proofreading tends to maintain a relatively high mutational rate, whereas the extreme compactness of the genome will prevent a large degree of genetic variability from occurring.

Subtypes

The first report of variability in HBV came from Le Bouvier (1971) who described two mutually exclusive subtype determinants, *d* and *y*. These reside in the surface protein together with the main antigenic determinant *a* (Levene & Blumberg, 1969). Two additional determinants, *w* and *r*, were described by Bancroft *et al*. (1972), who found that each HBV strain could be characterized as belonging to either subtype *adw*, *adr*, *ayw* or *ayr*. In a large study, additional subtypes were characterized by Couroucé-Pauty *et al*. (1983). The nine subtypes described were *ayw*1 to *ayw*4, *ayr*, *adw*2, *adw*4, *adrq*− and *adrq*+. A geographical pattern for the distribution of subtypes was confirmed.

Table 1. Direct relationship between the main antigenic subtypes of HBV and amino acid identity (Lys or Arg) at two positions (120 and 160) in the S protein

Amino acid 122 identity	Amino acid 160 identity	
	Lys	Arg
Lys	*dw*	*dr*
Arg	*yw*	*yr*

During the 1980s, it became increasingly clear that the subtype determinants are specified by one single amino acid, at positions 122 (*d* or *y*) and 160 (*r* or *w*) in the S protein, respectively (Okamoto *et al*., 1987b, c; Ashton-Rickardt & Murray, 1989a, b; Norder *et al*., 1991). Subtype determinants *d* and *w* have a lysine at both positions, whereas an arginine at both positions indicates subtype determinants *y* and *r* (Table 1). Additional subtype determinant reactivities have

Table 2. Relationship between HBV genotypes and subtypes

Those subtypes shown within parentheses are seldom seen in the genotype specified.

Genotype	A	B	C	D	E	F	G
Associated subtypes	*adw2* (*ayw1*)	*adw2* *ayw1*	*adr* *adrq* — *ayr*	*ayw2* *ayw3* *ayw4*	*ayw4* (*adw2*)	*adw4q* —	*adw2*

been mapped to amino acid positions 127, 144, 145, 158, 159, 177 and 178 (Okamoto *et al*., 1989; Norder *et al*., 1992a).

Subtyping of HBV strains was used for epidemiological purposes and, in some cases, to trace nosocomial chains of infection or to find correlations between disease and a particular subtype. Over the last decade, however, subtype determination has gradually been replaced by genotyping.

Genotypes, history and classification

In 1988, Okamoto *et al*. (1988) first suggested that the traditional subtypes could be complemented or replaced by a classification of different HBV strains into genetic subgroups. Comparing the full nucleotide sequences of 18 HBV strains, they found that these clustered into four groups, A to D, with more than 8% divergence between the groups. This degree of divergence has since become the definition for HBV genotype. The correspondence between subtypes and genotypes is shown in Table 2.

Using the polymerase gene instead of the whole genome, Orito *et al*. (1989) also found four separate subgroups, differing slightly from the groups described by Okamoto *et al*. (1988). Comparisons of S gene sequences were done by Norder *et al*. (1992b). In addition to results similar to those by Okamoto *et al*. (1988), two more groups, E and F, were described. In a larger study, they compared the S gene sequences from 122 strains and confirmed the existence of the two new groups (Norder *et al*., 1993b). A short region of the preS1 gene was studied by Uy *et al*. (1992). When studying the protein sequences of 33 HBV strains, Ogata *et al*. (1993) found a genotypic pattern that agreed with the results of Okamoto *et al*. (1988).

An extended study, where the complete nucleotide sequences of several strains were compared, showed that the previously described group E found in West Africa, although closely related to group D, constituted a genotype of its own (Norder *et al*., 1994). The year before, a highly divergent (15%) strain from Brazil had been reported (Naumann *et al*., 1993). It expressed the *adw*4 phenotype and constitutes genotype F. It has often been used as an outgroup in phylogenetic studies of HBV, as it is the most divergent human-derived genotype reported. Recently, Stuyver *et al*. (2000) described an additional genotype, G (Table 2).

Non-human hepadnaviruses

The first suggestion of the existence of HBV-like viruses among other species came from the Philadelphia Zoo, where eastern woodchucks (*Marmota monax*) had an unusually high occurrence of chronic liver disease and hepatocellular carcinoma (HCC). Virus-like particles that resembled HBV morphologically and had a weak cross-reactivity with HBsAg were found in the serum of these animals (Summers *et al*., 1978). The woodchuck hepatitis virus (WHV) has since been characterized and its genome cloned and sequenced (Galibert *et al*., 1982). There is 70% nucleotide identity between HBV and WHV and the organization of the WHV genome is almost identical to that of HBV (Tiollais *et al*., 1985).

A third mammalian hepadnavirus (GSHV) was found in California Beechey ground squirrels (*Spermophilus beecheyi*) (Marion *et al*., 1980). Its genome is more related to WHV (82% nucleotide identity) than to HBV (55% homology) (Tiollais *et al*., 1988). More recently, another ground squirrel hepatitis virus, this time found in wild arctic ground squirrels (*Spermophylus parryi kennicotti*) in Alaska, showed approximately 84% identity to GSHV as well as WHV and was designated arctic squirrel hepatitis virus (Testut *et al*., 1996). The death of a woolly monkey (*Lagothrix lagotricha*) from the Louisville Zoological Gardens from fulminant hepatitis led to the discovery of woolly monkey HBV (WMHBV). Among the orthohepadnaviruses, this virus is most closely related to HBV, the largest variability being seen between preS1 regions (Lanford *et al*., 1998).

The first avian hepadnavirus to be identified was the duck HBV (DHBV) infecting Pekin ducks (*Anas platyrhynchus*) in China (Zhou, 1980) and the USA (Mason *et al*., 1980). It is highly divergent from the other hepadnaviruses, with only about 40% nucleotide identity to HBV (Tiollais *et al*., 1988). Related to DHBV, but diverging 22% in nucleotide sequence, the heron HBV (HHBV) was found in German grey herons (*Ardea cinerea*) by Sprengel *et al*. (1988). Stork HBV was recently isolated and appears most closely related to HHBV (Pult *et al*., 2001b).

The host range of all hepadnaviruses is narrow. DHBV has been shown to infect geese and GSHV has been transmitted to woodchucks, but HHBV could not be shown to induce infection in ducklings (Sprengel *et al*., 1988). HBV can infect

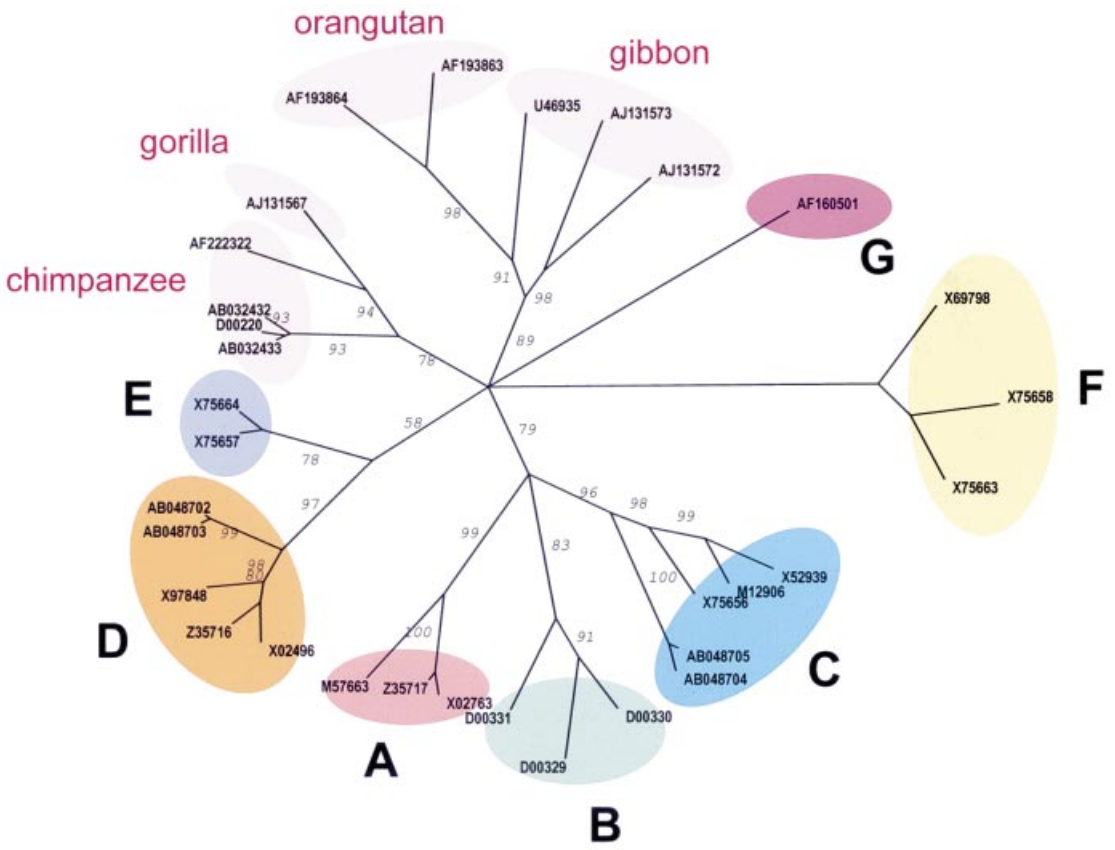

Fig. 2. Unrooted maximum-likelihood phylogenetic tree showing relationships between representative HBV strains derived from primates. All strains are labelled with their corresponding database accession number. Human-derived HBV strains belonging to different genotypes (A to G) are differentiated by colour. The outgroup used was genotype F strain X75658. WMHBV (accession AF046996) cannot be shown at this scale because of phylogenetic distance but branches off one-third of the way along the main genotype F branch and is more than twice as long as the latter. The tree and support values for internal branches are derived from the quartet puzzling program TREEPUZZLE (www.treepuzzle.de).

chimpanzees and some other primates but there also appear to be separate non-human primate genotypes within HBV.

The HBV strain from a persistently infected chimpanzee in the London Zoo was characterized and shown to diverge by about 10% from other human HBV strains (Vaudin *et al.*, 1988). Recently, there have been a large number of reports about variant HBV strains isolated from different primates. Serum from a chimpanzee that had been inoculated with serum from a white-handed gibbon (*Hybolates lar*), infected in the wild, showed the presence of an HBV genome most closely related to the chimpanzee strain described by Vaudin *et al.* (1988) (Norder *et al.*, 1996). Phylogenetic analysis suggested that the reported non-human HBV strains were indigenous to their respective hosts and not acquired recently. An extended study by Lanford *et al.* (2000) confirmed the existence of the gibbon HBV as a separate group within the human hepadnaviruses but suggested that the gibbon strains had diverged recently from the human HBV strains. The strains isolated from orangutans (*Pongo pygmeaeus*) in captivity and also in the wild (Warren *et al.*, 1999; Verschoor *et al.*, 2001) are more distantly related to human HBV. Two independent reports on wild chimpanzee strains from West Africa (Hu *et al.*, 2000; MacDonald *et al.*, 2000) found a close relationship with the strain above described from the London Zoo. A strain isolated from a captive gorilla (*Gorilla gorilla*) originating from Cameroon clustered with the chimpanzee strains (Grethe *et al.*, 2000). Relationships between representative primate HBV strains are illustrated in Fig. 2.

Structural differences between hepadnavirus genomes

The major genomic structural differences between members of the hepadnavirus family exist between mammalian and avian hepadnaviruses. DHBV DNA is almost fully double-stranded, whereas the mammalian hepadnaviruses have only partially double-stranded DNA. The latter have two presurface regions, preS1 and preS2, whereas the avian viruses only have one preS region. No X-gene has been found in DHBV and HHBV, although a recent report by Chang *et al.* (2001) describes a regulatory X-like protein from a hidden ORF in the DHBV genome. Table 3 compares the main genome characteristics of the different members of the *Hepadnaviridae*.

Table 3. Differences between hepadnaviruses

	HBV	WHV	GSHV	DHBV
Approx. genome size (kb)	3·2	3·3	3·3	3·0
Host	humans, primates	woodchucks	ground squirrels, woodchucks, chipmunks	ducks, geese
No. of surface proteins	3	3	3	2
X gene	present	present	present	absent

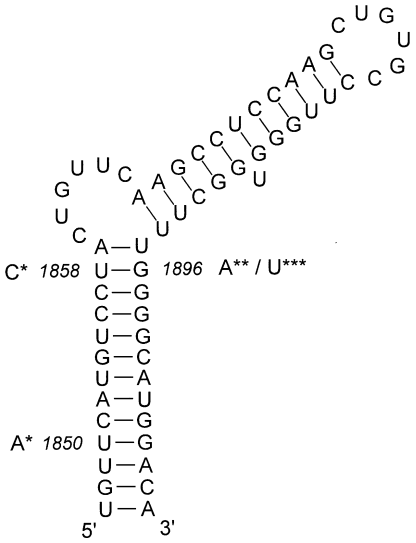

Fig. 3. The RNA stem–loop structure of the HBV encapsidation signal. Genotype-specific changes, *; mutation leading to a translational stop, **; normally occurring nucleotide in non-human HBV strains, ***.

As described above, HBV includes a number of non-human strains isolated from primates, which cluster into separate genotypes of HBV. Their genome organization is identical to that of HBV and they share the subtypic antigenic determinants of the human HBV strains.

It is noteworthy that amongst HBV strains belonging to HBV genotype D, there is a 33 nt deletion in the preS1 region (Heermann & Gerlich, 1991). No such deletion has been seen in other genotypes, even in members of the more closely related genotype E (Norder *et al.*, 1994; Bowyer *et al.*, 1997).

One salient feature of all hepadnaviruses is the secondary structure formed by the pregenomic RNA in the precore core region. Due to the ca. 130 nt terminal redundancy in pregenomic RNA, there are two copies of this structure, one at each end of the genome. The 5′ version of this stem–loop structure forms the encapsidation signal ε, which directs the packaging of pregenomic RNA into immature core particles during replication. Although there is a considerable sequence variation between different hepadnaviruses, they all form a stable stem–loop structure in this region and there is a large degree of sequence conservation within the different hepadnavirus groups. The stability of the stem–loop structure depends on strict conservation of base pairing in the stem region; mutations disrupting base pairing may lead to less efficient replication or non-viable virus particles. It is interesting to note that a G to U nucleotide change in the distal part of the lower stem, changing the sequence ^{1893}UUUGGGG^{1899} to UUUUGGG is seen in non-human HBV strains isolated from chimpanzees, orangutans, gorilla and gibbon, but also shared by WMHBV, a non-HBV strain (Fig. 3). This would affect the stability of the stem–loop structure in a similar way as does the G to A mutation at position 1896 in HBV genotype A strains (Li *et al.*, 1993).

Members of HBV genotype A and some members of genotype F carry a C at position 1858. This would lead to a stem-disruption if the ^{1896}G to A mutation occurs, leads to a translational stop and prevents the production of hepatitis B e antigen (HBeAg). This has also been reported for some genotype C strains (Alestig *et al.*, 2001). Computer modelling has predicted the effects of these changes on the pregenomic secondary structure of both the precore and the upstream core promoter regions (Kidd & Kidd-Ljunggren, 1996). Interestingly, it has been shown that this stop mutation can occur in genotype A strains. By analysing sequential samples, it has been shown that, in these cases, ^{1858}C first changes to a U, followed by the ^{1896}G to A mutation, thus protecting the base-pairing of the stem (Li *et al.*, 1993; Bläckberg & Kidd-Ljunggren, 2000a).

The phenomenon of RNA splicing in HBV has been known for some time (Günther *et al.*, 1997). In a recent study of spliced HBV genomes by Sommer *et al.* (2000), the authors found that minor splice variants differed between genotypes, implying that some minor splice sites are active only in certain genotypes. The same authors support the suggestion put forward by others (Rosmorduc *et al.*, 1995; Soussan *et al.*, 2000) in that splicing events may contribute to the persistence of HBV.

In a study where the secondary structure of the whole pregenomic RNA was predicted by computer modelling, some differences in RNA folding between genotypes could be seen (Kidd-Ljunggren *et al.*, 2000).

Distribution of HBV genotypes

The geographical pattern seen in HBV subtype distribution is even more distinct when the corresponding genotype prevalence in different geographical regions of the world is studied. HBV genotype A is mainly found in Northwestern areas of Europe and North America (Norder *et al*., 1993b). Some genotype A strains have also been found in the Philippines (Norder *et al*., 1993b; Kidd-Ljunggren *et al*., 1995), possibly reflecting the close contact with North America, especially over the last century. A similar epidemiological link would explain the genotype A strains isolated from patients in Hong Kong (Lok *et al*., 1994) and South and Eastern Africa (Bowyer *et al*., 1997). An analysis of the genotype A strains found in South Africa demonstrated that the majority clustered into a separate group within genotype A, called A′. The differences between A and A′ seemed to lie mainly in the preS2 region (Bowyer *et al*., 1997).

Genotype B and C strains belong in the indigenous population of Southeast Asia (Okamoto *et al*., 1988; Kidd-Ljunggren *et al*., 1995; Theamboonlers *et al*., 1999). Their distribution is fairly intermixed, with a tendency toward more genotype C strains being found in the Northern mainland regions and in mainland Japan (Orito *et al*., 2001a). However, genotype C especially is also found in the populations of the South Pacific islands, where the prevalence of HBV carriers is sometimes very high (Gust, 1984). Interestingly, it is possible to differentiate genotype C strains geographically by subtype. The genotype C strains isolated from the Pacific islands are more often of the *adrq* subtype, as compared to those strains from Southeast Asia (Norder *et al*., 1993b).

Genotype D is the most widely distributed genotype and has been found universally, with its highest prevalence in a belt stretching from Southern Europe and North Africa (Norder *et al*., 1993b; Borchani-Chabchoub *et al*., 2000) to India, in West and South Africa (Bowyer *et al*., 1997), and among intravenous drug users on all continents (Kidd-Ljunggren *et al*., 1999; Bläckberg *et al*., 2000; Flodgren *et al*., 2000). Genotype E is the most similar to genotype D genetically (Norder *et al*., 1993a, b, 1994) and has been interpreted as a subset of genotype D when using the X gene for phylogenetic analysis (Kidd-Ljunggren *et al*., 1995). It is found in West and South Africa and one of the main differences from genotype D is that it does not have the 33 nt deletion at the beginning of the preS1 region which is common to all genotype D strains (Bowyer *et al*., 1997; Norder *et al*., 1994; Odemuyiwa *et al*., 2001).

The most divergent genotype, F, is found in South and Central America (Norder *et al*., 1993a; Arauz-Ruiz *et al*., 1997a, b; Blitz *et al*., 1998; Mbayed *et al*., 1998; Nakano *et al*., 2001). Although it shares some structural features with genotype A strains (see above), it is believed to be the original genotype of the New World. It shows less homology than the other genotypes to the different primate strains that have been described. Genotype G has been found in France and the USA (Stuyver *et al*., 2000) but not in Japan (Kato *et al*., 2001).

In many countries where well-known waves of migration have occurred over time, the prevalence of different HBV genotypes reflects the origin of the immigrants and other patterns of migration. This is exemplified by South Africa, where the most prevalent genotypes, A and D (Bowyer *et al*., 1997), correlate with migration from Northwestern Europe (UK and the Netherlands), Southern Europe and India. The same genotypes in Argentina, A and D (Mbayed *et al*., 1998), reflect migratory waves from Northwestern Europe, Italy and Spain. In New Zealand and Australia, the same genotypes feature strongly, together with a number of genotype C strains contributed by immigrants from Southeast Asia and the Pacific Islands (Kidd-Ljunggren *et al*., 1995; Sugauchi *et al*., 2001). In a study of Belgian children who had received interferon treatment for chronic HBV infection, a child originating from Haiti harboured a genotype E strain (Liu *et al*., 2001). As HBV genotype E strains are found exclusively in West and South Africa, this provides a parallel to the chain of events reported for the transmission of human immunodeficiency virus (HIV) to the New World. Another unexpected genotype E infection was found in a Swedish sailor with acute HBV who had received a vitamin injection in West Africa 3 months earlier (unpublished data).

Not only migration but also behavioural patterns may change the prevailing genotype in a given region. In a recent study by Koibuchi *et al*. (2001), Japanese homosexual men coinfected with HIV were unexpectedly found to harbour HBV genotype A instead of C or B, which are the prevailing genotypes in Japan.

Mutation rate and evolution pattern of HBV

An accurate knowledge of the mutation rate of the hepadnaviruses could be of assistance in estimating when the different viruses and their genotypes branched off from each other. The first study attempting to estimate the mutation rate of HBV was reported by Okamoto *et al*. (1987a). By comparing different HBV clones isolated from the serum of a supposedly neonatally infected 54-year-old woman, the estimated mutation rate was found to be between 1·4 and $3{\cdot}2 \times 10^{-5}$ substitutions per site per year. This mutation rate is larger than that for DNA viruses and more similar to certain RNA viruses. When analysing the rate of substitutions in further depth, Orito *et al*. (1989) found the rate of synonymous (silent) substitutions to be approximately 5×10^{-5} per site per year, which places the mutation rate of HBV between those of DNA viruses and retroviruses. It is interesting to note in this context that they found a higher rate of synonymous than of non-synonymous substitutions in all four ORFs, signifying a constraint of the HBV genes by amino acid changes. Conflicting results were presented by Hannoun *et al*. (2000a), where non-synonymous substitutions constituted 88 % of the changes

observed in patients who, presumably, had been infected vertically.

The rate of substitutions *in vivo* depends on a number of factors, which are both separate and interdependent of each other. Thus, the conflicting virus strategies of a compact genome and replication through reverse transcription combine with host factors, such as immune response, and also with the risk of mutations arising from antiviral treatments. The host response appears to be an important factor, as there is evidence that the mutation rate over many decades is negligible in silent or occult HBV infection, where there is minimal host response (Bläckberg & Kidd-Ljunggren, 2000b). In a survey of Australian HBV-carrying schoolchildren, some of whom had been followed for 2 years, no sequence variations were found over time (McIntosh *et al*., 1998), which implies that there may not be much change in healthy carriers either. Preliminary results from a family where a carrier mother transmitted the infection to all of her five children, showed that there was no sequence variation between the HBV strain of the mother and those of the children, two of whom were identical twins aged 8 years (unpublished data). The study of the evolutionary rate of a viral genome in patients with genetically identical immune responses may partly eliminate one of the confounding factors present when assessing *in vivo* mutation rates.

The evolutionary rates of other hepadnaviruses, namely WHV and DHBV, have been studied experimentally (Girones & Miller, 1989; Argentini *et al*., 1999; Pult *et al*., 2001a). By measuring the number of revertants of a cytopathic DHBV strain injected into ducklings, a mathematical model was used to estimate the number of substitutions to between 0·8 and $4{\cdot}5 \times 10^{-5}$ per site per generation (Pult *et al*., 2001a), which approximates the mutation rate for HBV suggested by others (Okamoto *et al*., 1987a; Orito *et al*., 1989).

There have been several recent attempts to analyse the evolutionary history of HBV, both in terms of the origin of HBV and the time point when it diverged from the other hepadnaviruses (Norder *et al*., 1996; Warren *et al*., 1999; Lanford *et al*., 2000; Takahashi *et al*., 2000) and also from the point of divergence between HBV genotypes (Mizokami *et al*., 1997; Bollyky & Holmes, 1999). MacDonald *et al*. (2000) have summarized the attempts by several authors to determine the historical relationship of human and non-human HBV strains and suggested that a much larger number of both primate strains and human HBV strains, from poorly investigated areas with high endemicity, need to be analysed before any firm conclusions can be made.

Highlighting the significance of the overlapping ORFs for the mutation rate of HBV, Mizokami *et al*. (1997) proposed the term *constrained evolution* for the evolution of HBV. Due to the variability of substitution rates observed in an in-depth phylogenetic analysis of a large number of complete genomes and S gene sequences, no reliable molecular clock for the development of the HBV genome could be obtained and the origin of HBV remains obscure (Bollyky & Holmes, 1999).

Recombination

Unless whole genomic sequences are used, differentiation of HBV strains into separate genotypes will depend on which part of the genome is used in the phylogenetic analysis. The rooted phylogenetic trees obtained by comparing different HBV genes from a number of strains differ considerably and illustrate the importance of selecting the appropriate regions for analysis (Norder *et al*., 1994). One reason for such large variability in the trees obtained is recombination (Robertson *et al*., 1995a, b). Recombination, although not genotype-related, was reported from HCC-associated HBV (Georgi-Geisberger *et al*., 1992). When analysing individual ORFs and complete genomes, respectively, from a number of HBV strains, Bollyky *et al*. (1996) found evidence for recombination in two strains. In both cases, the mosaic strains originated from geographical areas where several genotypes are known to exist. Several additional studies support the theory of recombination between genotypes that co-circulate in some geographical regions, such as genotype B/C switching and genotype A/D switching (Bowyer & Sim, 2000; Morozov *et al*., 2000; Owiredu *et al*., 2001). Two studies analysing Vietnamese strains found recombination between genotypes C and A (Hannoun *et al*., 2000b) and B and C (Yuasa *et al*., 2000), respectively. It is still unclear, however, how these events of recombination have arisen in HBV. Bearing in mind the extreme compactness of the HBV genome and the strategy of replication, with a single, particle-associated RNA genome converting into a partially double-stranded DNA virus, the probability of a template switch appears very low (Georgi-Geisberger *et al*., 1992). However, it would be difficult to refute that, whatever the original mechanism, recombination in a host arises either from simultaneous transmission of several genotypes or from sequential infections with different genotypes.

Clinical differences between HBV genotypes

The clinical course of infection with HBV varies, depending on the one hand on the patient's age and immune response and, on the other hand, as increasing evidence is showing, on the virus strain infecting the individual. Overall, less than 1% of acute infections lead to fulminant hepatitis and death. Approximately 0–10% of infected adults become chronic carriers of HBV (Sherlock, 1985; Bläckberg *et al*., 2000). Perinatal transmission leads to up to 90% of chronic carriership. Chronic carriers often lack symptoms but may have histological evidence of hepatocellular damage from mild inflammation to cirrhosis and HCC.

Early studies demonstrating subtype-related clinical differences include the association of Gianotti's disease with subtype *ayw* in Japan (Ishimaru *et al*., 1976) and a higher frequency of liver dysfunction in *adr*-infected patients compared to those infected with *adw* (Shiina *et al*., 1991a, b; Noguchi *et al*., 1994). Taking into account that genotype C strains

are most often of subtype *adr*, the latter results have been confirmed by several studies of Southeast Asian chronic carriers (Lindh *et al*., 1999; Ding *et al*., 2001; Orito *et al*., 2001b).

The correlation between chronic HBV infection and HCC has been proven both epidemiologically (Beasley *et al*., 1981) and experimentally (Brechot *et al*., 1980). Most large-scale reports of HCC and HBV have come from Southeast Asia, reflecting the high prevalence of chronic HBV infection in this region of the world. In a recent study by Kao *et al*. (2000a), a large number of Taiwanese HCC patients was studied and compared to patients with cirrhosis and asymptomatic carriers. These authors found that genotype C was more common in cirrhotic patients. There was also a striking difference in genotypes found in HCC patients, depending on the age of the patient. In HCC patients older than 50 years, genotype C was the most prevalent, whereas in patients younger than 50 years, genotype B was the most common. This difference was even more pronounced in the HCC patients under 35 years in whom no genotype C was found. A possible explanation would be that genotype C infection leads to HCC through cirrhosis. These results are partly in conflict with those described by Orito *et al*. (2001a) from Japanese patients, where genotype B infected patients with HCC were older than HCC patients with genotype C. In a prospective study where a large number of cirrhotic patients was followed, genotype C-infected patients developed HCC more frequently than genotype B-infected patients. The genotype C-associated HCC was also more resistant to treatment (Tsubota *et al*., 2001).

There have been few reports of genotype correlations in fulminant hepatitis. It is notable that von Weizsäcker *et al*. (1995) found heterogeneous virus populations in sera from three carrier mothers who transmitted neonatal fulminant hepatitis to their babies. Subtypes *adw2* and *ayw* were present simultaneously in the mother's sera. In one baby who survived, both subtypes were transmitted. In the two babies who died, only subtype *ayw* could be detected. Analysing strains from fulminant hepatitis patients in Vietnam, Yuasa *et al*. (2000) found that they mostly belonged to genotype B and that fulminant genotype B strains differed from non-fulminant strains by a specific mutation in the X gene. Outbreaks of fulminant virus hepatitis in some parts of the world have been associated with concomitant infection with hepatitis D virus (HDV). HBV genotype F together with HDV genotype 3, seen in outbreaks in Northern South America, is believed to be more highly correlated with the development of fulminant hepatitis. However, in a recent study of fulminant hepatitis in Samara, Russia, the prevailing strains were HBV genotype D and HDV genotype 1 (Flodgren *et al*., 2000).

In a cross-sectional study by Mayerat *et al*. (1999), genotype A was suggested to lead more often to chronicity as it was found more often in chronic hepatitis patients than genotype D, whereas the opposite situation was found in patients with acute hepatitis. An overrepresentation of drug addicts in the acute case group could well explain the higher prevalence of genotype D, the genotype predominantly infecting intravenous drug users in the Western world. In another study, genotype D was found to be associated with more severe disease in post-transplant patients with recurrence of HBV infection (McMillan *et al*., 1996). However, most of the genotype D strains described had single or double mutations at the end of the precore gene (^{1896}G to A and ^{1899}G to A). These mutations have been associated with more severe disease and have been found in many studies where strains leading to fulminant hepatitis were analysed. In order to minimize the influence of confounding factors when interpreting results such as these, large-scale studies are necessary. Ideally, a prospective study where neonatally infected babies infected by genotype A, B, C, D, E, F or G were followed until adulthood would give an answer to the question of genotype differences in the long-term outcome of HBV infection.

Interferon, which has been used to treat HBV infections, was shown to give better response in patients from Northwestern Europe than in vertically infected patients from Southeast Asia (Thomas *et al*., 1987). It is quite possible that the origin of the patient plays a low role in these observations and that the differences rather reflect the HBV genotypes prevailing in these separate geographical regions. Two recent studies support this theory. A retrospective analysis of the results of interferon treatment in chronic carriers (genotypes B and C) demonstrated that genotype C had a lower response to interferon (Kao *et al*., 2000b). Treatment with nucleoside analogues has largely replaced interferon. Patients infected with *adw* strains had a 20-fold increased risk of lamivudine-resistance than patients infected with *ayw* strains (Zollner *et al*., 2001).

Serological and genotypic shift

Genotype differences and genotype shifts correlated to seroconversion have been reported from different groups. Seroconversion from HBeAg to anti-HBe has been believed to be associated with either the emergence of a translational stop codon in the precore gene (^{1896}G to A mutation), precluding the expression of HBeAg (Carman *et al*., 1989) or the appearance of a double mutation (^{1762}A to G and ^{1764}G to A) in the upstream core promoter, regulating the transcription of the precore gene (Okamoto *et al*., 1994). In many cases, both changes have been seen in anti-HBe-positive patients. Although there have been numerous cross-sectional studies analysing these mutations, little has been known about the temporal sequence of seroconversion correlated to the appearance of mutations. In a study of Chinese patients in Hong Kong, 92% of the samples showed precore and/or core promoter changes after seroconversion to anti-HBe, thereby implying a definite role for these mutations in seroconversion (Chan *et al*., 1999). Different results were obtained from a study of patients in Sweden, where only 50% of the strains showed

any mutations after seroconversion (Bläckberg & Kidd-Ljunggren, 2000a). The difference may be explained by the distribution of genotypes in both studies. Although all samples were not genotyped in the Chinese study, the 11 strains in which this was performed belonged to genotype B or C. In the Swedish study, where genotypes A to E were represented, genotypes A and D were by far the most common and HBeAg seroconversion was confirmed to occur *earlier* than core promoter or precore mutations in genotype D.

That seroconversion from HBeAg to anti-HBe and from HBsAg to anti-HBs can lead to change of genotype in the infected patient has been reported in several studies by the one group. In a small group of chronic carrier children who remained HBV DNA-positive while they became serologically HBsAg-negative, three showed a change from subtype determinant *d* to *y* (Bahn *et al.*, 1997). In two other studies, children who seroconverted to anti-HBe changed their HBV genotype from A to D in seven cases and from D to A in three cases (Gerner *et al.*, 1998; Friedt *et al.*, 1999). This latter change was also seen in a neonatally infected baby with fulminant hepatitis who survived. It is not clear how a complete genotypic change would occur in the one patient, unless the patient had originally been infected with more than one genotype and an immune selection occurred during seroconversion.

Nosocomial infections

Characterization of HBV strains by subtype or genotype has been used to investigate chains of infection in different settings. Using the X and S genes, Hawkins *et al.* (1996) linked an outbreak of acute HBV with subtype *adw* in a haematology unit to contamination of a cryopreservation tank storing bone marrow. By subtype comparison, a large number of silent HBV infections among the members of an American football team were traced to one member of the team who was a chronic carrier of HBV subtype *adr* (Tobe *et al.*, 2000). By finding the same genotype, and by further sequence analysis, identical strains in two elderly women and a number of intravenous drug users with acute HBV infection, a likely transmission through multiple-dose vials could be implicated (Kidd-Ljunggren *et al.*, 1999). In an unusual chain of events, a neonatally infected infant transmitted the infection to two paediatricians who both died from fulminant hepatitis (Kosaka *et al.*, 1991). The strains were found to be identical and were subsequently used to transmit the infection to a chimpanzee (Ogata *et al.*, 1993).

Subtyping and sequencing of a region of the core gene were used to show the transmission of HBV from a thoracic surgeon to several patients on whom he had operated (Harpaz *et al.*, 1996). The risk of nosocomial infection with HBV during thoracic surgery is further highlighted by two studies on heart-transplant patients. In Hannover, *ayw2* was found in previously HBV-susceptible patients who had undergone a heart transplant (Petzold *et al.*, 1999). When the whole genomes were sequenced, they were found to differ by 18 nucleotides from the most similar published strain. Osterhaus *et al.* (1998) found one strain, *ayw2*, in 20 of 21 heart transplant recipients in Rotterdam. Additional support for a nosocomial infection in such cases is the low prevalence, less than 10%, of subtype *ayw2* among the HBV-infected patients in the Netherlands. Knowledge of the prevalence of HBV genotypes in our local setting made it more likely that an acute HBV infection in a kidney transplant patient was caused by reactivation rather than by a new infection (Kidd-Ljunggren & Simonsen, 1999). Haemodialysis centres were found very early on to be at risk for nosocomial HBV transmission (Löfgren *et al.*, 1982), both between patients and from patients to staff. A recent study from a city in Brazil by Teles *et al.* (1999) shows that this problem still persists. In their survey of patients attending haemodialysis centres, two centres were shown to harbour exclusively infection with genotype D, subtype *ayw2*, whereas three centres showed predominance of genotype A, subtype *adw2*.

Test methods

When genotypic classification of HBV came into use, the first reports were based on complete genome sequences (Okamoto *et al.*, 1988; Orito *et al.*, 1989). Gradually, the sequences of single genes or parts of genes were used in order to facilitate the comparison of a larger number of strains.

The search for methods that would not have to involve sequencing led to the technique of restriction endonuclease analysis of HBV PCR products, also called restriction fragment length polymorphism (RFLP) (Shih *et al.*, 1991; Niel *et al.*, 1994; Lindh *et al.*, 1997, 1998; Mizokami *et al.*, 1999). Often, the S gene or a combination of the preS and S genes have been used. In the study by Mizokami *et al.* (1999), a comparison of RFLP results from full-length genomes with those from S genes demonstrated that using the S gene alone could be accurate enough to differentiate between the six genotypes A to F. Another method which is also based on further analysis of PCR products is known as post-PCR hybridization or line probe assay (Grandjacques *et al.*, 2000). Naito *et al.* (2001) recently described a PCR discriminating between different genotypes by using genotype-specific primers.

Taking advantage of the genotypic variability of the preS2 gene, Usuda *et al.* (1999) raised monoclonal antibodies to genotype-specific epitopes in this region and developed an ELISA discriminating between genotypes A to F (Usuda *et al.*, 2000).

Conclusions and future perspectives

It took 36 years from the first report of the Australia antigen until the HBV from Australian aborigines had been characterized phylogenetically (Sugauchi *et al.*, 2001). Mainly during the latter third of this period, a substantial knowledge about HBV variability has been compiled. A number of

additional members of the *Hepadnaviridae* family have been characterized and several non-human HBV strains have been found.

We have seen that the strict geographical pattern of HBV genotype prevalence has shifted, especially in areas of the world where more migration has occurred. It is probable that this geographical pattern will become even more loose with increases in migration. It is also possible that the events of recombination between genotypes that have been reported lately (A with D, A with C and B with C) will increase as different genotypes circulate in the same region. There are structural and clinical differences between genotypes and it remains to be seen whether recombination will create strains with other, or even larger, differences between them.

The most thoroughly analysed region of the HBV genome in the context of structural differences between genotypes is the precore region, where the stability of the secondary structure formed by pregenomic RNA depends on strict base-pairing, which differs between some genotypes. The importance of this structure for the replication of HBV is undisputed; however, few studies have concentrated on the importance of structures elsewhere in the genome and the impact of genotypic differences on these. Both at the nucleic acid and at the protein levels, genotypic variability may lead to structural changes which could have far-reaching effects. New techniques, such as nuclear magnetic resonance, will undoubtedly expand our knowledge about different HBV structures and genotypic changes within these. This will be important from a clinical perspective also, as many new antiviral compounds are targeted against specific structures in the replication process and the response rate may depend on variability of these structures.

Parts of the preS region constitute the most variable part of the HBV genome and this region is important for virus attachment and cell entry. Advances are currently being made in the development of cell culture systems to sustain HBV replication. It will be interesting to study differences between genotypes in infectivity and virus viability in these and future systems.

There is increasing evidence of clinical differences between subtypes and genotypes at various levels, including seroconversion age from HBeAg to anti-HBe, the risk for development of severe liver injury, including HCC, and the response to antiviral treatment. Unfortunately, and due to the geographical pattern of genotype distribution, most studies have compared genotype A with D or genotype B with C. Thus, no general consensus has appeared about the degree of virulence of different genotypes. It appears, though, as if genotype C, with its highest prevalence in Southeast Asia, may lead to more severe disease than some of the other genotypes. In this context, it is necessary to remember that the genetic make-up of the host may have a strong significance and may affect the long-term interactions between virus and host. This is an issue that has been studied in terms of vaccination success or failure but more studies about its effect on long-term HBV infection are needed.

There are many hundreds of hepadnavirus sequences in the databases. Despite many excellent studies using various phylogenetic methods to elucidate the history of hepadnaviruses, the origin of HBV is still obscure. It will be a strong challenge to try to answer some of the complex questions outlined above, and analysis of similarities and differences between strains will continue to form an integral part of this work.

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