Application of Hepatitis B Virus (HBV) DNA Sequence Polymorphisms to the Study of HBV Transmission

Hsiang Ju Lin, Ching-lung Lai, I. J. Lauder, Pui-chee Wu, Theodore K. Lau, and Ming-wei Fong

Clinical Biochemistry Unit and Departments of Medicine, Statistics, and Pathology, University of Hong Kong

Short sequences in hypervariable regions of the hepatitis B virus (HBV) genome can be used to identify different strains, providing a novel approach to the study of HBV transmission. The nucleotide sequence in positions 2551–2650 (1: *Eco*RI site) was determined for serum HBV DNA from 96 Chinese children living in Hong Kong and from 38 of their parents. HBV DNA was extracted and sequenced after amplification with the polymerase chain reaction, using as primers oligonucleotides corresponding to two conserved sequences. Among 82 unrelated children, 32 HBV DNA variants were present. One sequence was present in 33 children and 31 variants were found among the other 49. Siblings within each of nine families had the same variant; in three families siblings had different variants. Six of the eight fathers and 28 of the 30 mothers had HBV DNA sequences identical to those of their offspring. A total of 34 variants were found among the 134 individuals. The hypothesis of random assortment of sequences in parents and children was rejected ($P < .00005$). Thus, this new approach proves the occurrence of intrafamilial transmission of HBV among Chinese.

The modes of transmission of hepatitis B virus (HBV) differ in various parts of the world. In the United States, sexual activity and intravenous drug use are important risk factors [1] whereas perinatal HBV transmission is common in southeast Asia [2]. Studies of HBV transmission have been based largely on epidemiologic surveys, serologic tests, and clinical histories. In addition, intrafamilial transmission among Chinese has been demonstrated by serotyping [3] as has the clustering of infected individuals in certain families [4]. Such studies can show the prevailing mode of transmission among various populations, but generally do not provide sufficient detail to confirm or exclude the occurrence of HBV transmission between specific individuals.

We recently reported the finding of mutant HBVs in two Chinese families and presented DNA sequence analyses to show the most probable routes of transmission within each family [5]. We now explore the possibility that certain regions of the HBV genome can be used to identify HBVs and so elucidate the transmission of this virus between individuals. To utilize DNA sequences for identification of particular viruses, there is an obvious advantage in choosing a polymorphic region. The first complete sequence of HBV DNA appeared in 1979 [6]; 13 other sequences have since been published [7–18]. Analysis of these 14 genomes showed that the sequences from the end of gene C through the pre-S regions were two to three times more variable than the most highly conserved region, gene S. The sequences between gene S and region X were also highly variable. Further, because amplification and sequencing of DNA require primers, the chosen region had to be conveniently close to some conserved sequences that could be used as such. The selected target was located at positions 2551–2650, between the core gene and the pre-S region, bracketed by conserved sequences with 5′ ends at positions 2463 on the S(+) strand and 2839 on the L(−) strand. The 100 nucleotide sequence represented 3% of the HBV genome.

Subjects and Methods

The subjects were 96 children who carried HBV, 90 of whom participated in a clinical trial [19]. All 96 were hepatitis B e antigen (HBeAg)–positive. None had undergone blood transfusions, acupuncture, or tattooing. Blood specimens were also obtained from 97 parents and tested for HBV serologic markers by ELISA (Abbott Laboratories, North Chicago, IL). HBV DNA was extracted from 100-μl samples with the aid of proteinase K digestion (2.50 mg/ml), phenol-chloroform treatment, and ethanol precipitation. The polymerase chain reaction was done as previously described [5] with primers 2463, 5′d(CCTTGGACTCATAAGGTGGG), and 2839, 5′d(TCCCAAGAATATGGTGACCC), using an initial denaturation step of 2 min at 95°C, followed by 30 cycles of heating for 1 min at 94°, 56°, and 72°C. Under these conditions the 376-bp target sequence was amplified $>10^6$-fold. The products were detected by viewing ethidium bromide–stained gels with UV illumination. The sensitivity of the method was ~20 pg/ml of whole genomic HBV DNA, as judged by results in six separate runs on serum with no serologic markers for HBV, to which known amounts of cloned

Received 28 January 1991; revised 29 March 1991.

Presented in part: International Symposium on Viral Hepatitis and Hepatocellular Carcinoma, Hong Kong, December 1990.

Grant support: Roche Asian Research Foundation and University of Hong Kong (to H.J.L.).

Reprints or correspondence (present address): Dr. H. J. Lin, Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

The Journal of Infectious Diseases 1991;164:284–8

0022–1899/91/6402–0008$01.00

VARIANT NO.	HOMOLOGY (%)	S-STRAND: 5' TO 3'									NO. OF CARRIERS
1	100	CTGACATTCA	TTTGCAGGAG	GACATTGTTG	ATAGATGTAA	GCAATTTGTG	GGGCCCCTTA	CAGTAAATGA	AAACAGGAGA	CTAAAATTAA	TTATGCCTGC	33
2	99	-G---------	----------	----------	----------	----------	----------	----------	----------	----------	----------	1
3	99	----T-----	----------	----------	----------	----------	----------	----------	----------	----------	----------	1
4	99	----------	----A-----	----------	----------	----------	----------	----------	----------	----------	----------	1
5	99	----------	----------	----------	----------	----------	----------	----------	--C-------	----------	----------	2
6	99	----------	----------	----------	----------	----------	----------	----------	------A---	----------	----------	1
7	99	----------	----------	----------	----------	----------	----------	----------	----------	--------G-	----------	1
8	98	-G---------	----------	----------	--------C-	----------	----------	----------	----------	----------	----------	1
9	98	----------	----------	A---------	----------	----------	----------	----------	----------	--C-------	----------	1
10	98	----------	----------	---------A	----------	----------	----------	----------	--C-------	----------	----------	1
11	98	----------	----------	----------	---A------	----------	----------	-C--------	----------	----------	----------	1
12	98	----------	----------	----------	----------	----------	--A-------	-----C----	----------	----------	----------	1
13	97	-C---------	----------	----------	----------	----------	----------	----------	--C-------	----------	--T-------	1
14	96	----------	----------	----------	----G-----	----------	--A-----A-	----------	----------	T---------	----------	1
15	89	--C-------	----------	------A--A	--------C-	A----A----	--C--T----	----T-----	---A------	T---------	----------	1
16	88	--C-------	---A------	------A--A	--------C-	A----A----	--C--T----	----T-----	---A------	T---------	----------	1
17	86	--C-------	---A------	------A--A	--------C-	A----AC---	--C--T----	--A-T-----	---A------	T---------	----------	2
18	86	--C-------	---A------	------A---	-G------C-	A----AC---	--C--T----	--A-T-----	---A------	T---------	----------	0
19	83	--C-------	----A-A---	--T--CA-C-	--------C-	A----A---A	--C--T----	----T-----	---A------	T-------G-	----------	1
20	83	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--G----	----T--C--	---A------	T---------	----------	1
21	83	--C-------	----A-A---	--T---A--A	--------C-	A----A---A	--C--T----	----T--C--	---A------	T-------G-	----------	4
22	83	--C-------	----A-A---	--T---A--A	--------C-	A----A----	--C--T----	----T--C--	---A------	T-------G-	-C--------	1
23	82	--C-T-----	----A-A---	--T---A-CA	--------C-	A----A----	--C--T----	----T--C--	---A------	T---------	-C--------	1
24	82	--A-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--T----	----T--C--	---A------	T-------G-	----------	1
25	82	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--T----	----T--C--	---A------	T-------G-	----------	6
26	82	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--G----	----T--C--	---A------	T-------G-	----------	1
27	82	--C-------	----A-A---	--T---A-CA	--------C-	A----A----	--C--T----	----T--C--	---A------	T-------G-	-C--------	7
28	81	-GC-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--G----	----T--C--	---A------	T-------G-	----------	0
29	81	--C-------	---AA-A---	--T---A-CA	--------C-	A----A----	--C--T----	----T--C--	---A------	T-------G-	-C--------	3
30	81	--C-------	----A-A---	--T---A-CA	-G------C-	A----A----	--C--T----	----T--C--	---A------	T-------G-	-C--------	1
31	81	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--T--G-	----T--C--	---A------	T-------G-	----------	1
32	81	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--T----	-C--T--C--	---A------	T-------G-	----------	1
33	81	--C-------	----A-A---	--T---A-CA	--------C-	A----A----	--C--T----	----T--C--	--GA------	T-------G-	-C--------	1
34	81	--C-------	----A-A---	--T---A-CA	--------C-	A----A---A	--C--T----	----T--C--	---A--A---	T-------G-	----------	1

Figure 1. Variant sequences in positions 2551–2650 of hepatitis B virus genome in 82 families. The carriers refer to 82 unrelated children. Variants 18 and 28 were present in two of their fathers.

HBV DNA had been added. After recovery of the fragments from gels, the DNA was labeled with ^{32}P using primer 2463 and the S strand was sequenced using methods previously described [5]. Sequences were compared with the aid of the LFASTA program [20].

Results

To assess the relative frequency of particular sequence variants, we first considered the number of variants found among children who were unrelated to each other. Thirty-two different sequences were found in positions 2551–2650 among 82 unrelated children (figure 1). The frequency distribution of the sequences was heavily biased toward variant 1; 33 of the children carried this variant. Thirty-one other sequences were distributed among the 49 other children; the second most frequently carried sequence was found in 7 children. Twenty-five sequences were found in only 1 subject among the 82. Relative to variant 1, the homology of the other variants ranged from 81% to 99%.

All of the Hong Kong variants differed from published sequences except for variant 16, which was identical to those found in subtype *adr* HBV DNAs analyzed in Japan [9] and Korea [17]. The homology of variant 1 ranged from 73% relative to two HBV DNAs of subtypes *ayw* and *adyw* studied in Europe [6, 13] to 92% relative to a subtype *adw* sequence from an Indonesian subject [11].

Serum specimens were obtained from 97 parents (67 families). Based on the serologic markers, all but 2 of the 59 parents who had little or no circulating HBV showed evidence of past or on-going HBV infection (table 1). Thirty-eight specimens, mostly HBeAg-positive, had sufficient amounts of HBV DNA for sequencing. We thus obtained sequence data from the parents of 35 unrelated children.

We compared variant sequences in family members (table 2). Among the 30 mothers whose serum HBV DNA was sequenced, 27 (90%) had the same variant as their offspring. In family 14, where the siblings had different variants, the mother had the same variant as one of the two children. In families 13 and 22 the mothers had variants that differed from those in their offspring. Among the 8 fathers in whom the HBV DNA sequence was determined, 6 (75%) had the same variant as their offspring. Two of the fathers had variants not found in any of the other subjects, bringing the total number of variants among the 134 subjects to 34. There were 11 sibling pairs and one group of 3 siblings. In 9 families, siblings had the same variant; in the other 3 (14, 41, and 42) the variants differed.

Among these 134 subjects, variants 7, 11, 12, 16, 22, 26, 30, 31, and 33 occurred exclusively in two or more members of nine families. Another 12 sequences occurred in singleton among the children: numbers 3, 10, and 15 (parents' sera unavailable), 6, 9, 13, and 24 (HBV DNA undetected in both parents), numbers 14, 19, 20, and 23 (fathers' sera unavailable; HBV DNA undetected in mothers' sera). Variant

Table 1. Hepatitis B virus (HBV) serologic markers in 96 children who carried HBV and in 97 parents.

Subjects, HBV DNA testing	No.	HBsAg	HBeAg	Anti-HBe	Anti-HBs	Anti-HBc
Children, sequenced	96	+	+	NT	NT	NT
Parents						
Sequenced	32	+	+	NT	NT	NT
	5	+	−	+	NT	NT
	1	+	−	−	NT	NT
Insufficient for sequencing	1	+	+	NT	NT	NT
	5	+	−	+	NT	NT
Not detected	25	+	−	+	NT	NT
	19	−	NT	NT	+	NT
	3	−	NT	NT	−	+
	2	+	−	−	NT	NT
	1	+	−	+	NT	NT
	1	−	NT	NT	+	NT
	1	−	NT	NT	−	−
	1	−	NT	NT	−	NT

NOTE. HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, anti-HBs, and anti-HBc antigens = antibodies to hepatitis B e, surface, and core antigens, respectively; NT, not tested; +, positive, −, negative.

34 occurred in a child with an HBV DNA-negative father and a mother carrying variant 25.

Table 3 statistically analyzes the 35 families in which parental HBV DNA sequences were obtained. In these families, 33 (94%) of the offspring had at least one parent with an identical HBV DNA sequence. The calculation of exact probabilities for the observed outcomes under the hypothesis of random infection was confounded by the lack of DNA sequence data for some of the parents. We therefore assumed that all parents in these families were possible sources of HBV transmission. This assumption was reasonable since all but 1 parent in the 35 families had serologic evidence of HBV infection.

We calculated the probability (p_i) of an offspring with variant i (where i is any 1 of the 32 variants found in the children) having at least one parent with an identical sequence. The frequency (F_i) of variant i was calculated from figure 1 (for variant 1, $F_1 = 33/82$). Under the hypothesis of random infection p_i is given by $2F_i - F_i^2$. In the analysis of families 1–35 and 1–30 the p_i values were conservatively set at $p_1 = 2F_1 - F_1^2$ for all offspring, facilitating straightforward binomial calculation of *P* values with the probability p_1 of obtaining a match. Thus, the hypothesis of random assortment of sequences in the parents and children could be rejected conclusively ($P < .00005$).

Discussion

Spontaneous mutations and biologic pressures have shaped contemporary HBV DNA sequences. Figure 1 shows a pattern of nonrandom variation in which 65% of the nucleotides were nonvariant. Nucleotide positions 2553–2648 encoded amino acids 83–114 of gene P. Compared to the amino acid sequence encoded by variant 1, 14 of the substitutions found in the other variant nucleotide sequences were synonymous, while 18 other substitutions translated into different amino acids. No stop codons were formed. The mutation rate for HBV DNA has been estimated to be $1–5 \times 10^{-5}$ nucleotide substitutions per site per year, which is comparable to or lower than those shown by retroviruses, influenza, or human immunodeficiency viruses (HIV) [18, 21, 22] and is independent of the human host DNA mutation rate that is $\sim 10^{-9}$ [23]. Deficiencies in any one of the complex processes that accompany viral infection, such as organ tropism and viral replication in the face of host defenses against infection [24], would place biologic constraints on the replication of HBV mutants.

By identifying HBV DNA sequences among family members, we have conclusive evidence that intrafamilial transmission of HBV occurs in Chinese families. In the few families in which HBV sequences differed among members, we considered the extent to which the sequences diverged. The degrees of homology between the variant sequences that differed within a given family fell into two groups. In families 14, 22, 41, and 42 (table 2) HBV DNA sequences in mother-offspring pairs or sibling pairs differed by 1%. We excluded the possibility that such differences resulted from misincorporation of bases in the polymerase chain reaction, the extent of which has been estimated at 0.25% for the products of a 30-cycle amplification [25]. Two blood specimens drawn on different occasions from each of the children in families 14, 41, and 42 were analyzed; the HBV DNA sequences in the specimens from each subject were identical. The HBV DNA preparations from the mother and child in family 22 were sequenced at least twice with the same results. The observed differences could probably be explained by spontane-

Table 2. Variant hepatitis B virus (HBV) DNA sequences in family members.

Family	Father	Mother	Child	Sibling(s)
1	NA	1	1	1
2–9*	NA	1	1	
10	NA	7	7	7
11	NA	11	11	
12	NA	22	22	
13	NA	25	1	
14	NA	25	25	32
15	NA	31	31	
16	NA	33	33	
17–19	–	1	1	
20	–	2	2	
21	–	17	17	
22	–	25	34	
23–25	–	27	27	
26	–	29	29	
27	–	30	30	30
28	1	1	1	
29	18	1	1	
30	28	12	12	12
31	1	NA	1	
32	16	NA	16	
33, 34*	1	–	1	
35	27	–	27	
36*	NA	–	1	1
37, 38	NA	NA	1	1
39	NA	NA	26	26
40	NA	NA	29	29
41	NA	NA	2	8
42	NA	NA	1	1, 4

NOTE. NA, serum not available; variant sequence numbers refer to variants in figure 1; –, HBV DNA not detected.
* Includes members of two families studied previously [5].

ous mutation after infection or by heterogeneity of the HBVs at the source of infection. It is unlikely that in every such instance that mutations occurring after infection were the cause of the observed divergence. The number of nucleotides in a 100-nucleotide sequence that undergo substitution over a 10-year period is far less than 1 (~0.05). The divergence may be attributable to the heterogeneity of HBV DNA in many carriers, which is well documented [7, 26–28]. Nucleotide sequences in HBV DNAs cloned from a single carrier differ by no more than 0.5% [18, 26]. It is possible that HBV transmission from such carriers to different individuals would lead to infection with strains that showed slight differences in DNA sequence. Consequently, we did not exclude the possibility of intrafamilial spread of HBV in these cases. In contrast, the differences in HBV DNA sequences between parent and offspring in families 13, 29, and 30 were 18%, 14%, and 17%, respectively. We are confident that the mother in family 13 and the fathers in the other two families could not have been the sources of HBV infection in their offspring.

It is possible that intrafamilial transmission occurred in some of the 31 children with no parental HBV DNA available for analysis. Among Chinese women there is a sharp increase in seroconversion to anti-HBe at ages 21–30 years [29]. HBV transmission could have occurred from a mother who subsequently underwent seroconversion, which is usually accompanied by diminished circulating HBV DNA. It is also possible that these 31 children were infected by extrafamilial sources.

Identity within a 100-nucleotide sequence does not imply identity throughout the genome as proved by the occurrence of three sequence duplications in positions 2551–2650 among the 14 reported nonidentical genomes. One such duplication occurred among HBVs of different serologic subtypes [10, 18]. We know there was some heterogeneity among the genomes with variant sequence 1 because the two HBV DNAs with the highly atypical mutation in position 2735 were among them [5]. Nonetheless, the dominance of variant 1 was striking. It is difficult to assess the significance of this finding in the absence of other comparably large surveys of viral nucleotide sequence in different subjects. It could be temporal and confined to a geographic location. A parallel might be the finding of immunologically distinct strains within a subgroup of respiratory syncytial virus, one of which showed a temporary dominance within a particular locality [30].

In contrast to the mutability of HIV and some other viral nucleic acid sequences [22, 24], HBV DNA appears to be stable over relatively long periods. The same variant sequence was found in two generations of many families. In one family three generations carried the same mutant HBV DNA sequence [5]. We also studied 10 subjects by sequencing the HBV DNA in two blood specimens taken at intervals of up to 39 months (mean, 23.5 months) and found no changes.

There is sufficient variability in HBV DNAs within a population to use sequence polymorphisms to elucidate routes of HBV transmission. The sequencing of a short segment, such as a 100-nucleotide region, was useful as a screening procedure. Specific problems may require that HBV DNAs found to be identical over a short region would have to be characterized further by sequencing other regions. Analysis of HBV DNA sequence polymorphisms may have other applications,

Table 3. Statistical analysis of 35 families in which parental hepatitis B virus DNA sequences were obtained.

Families	Matching sequences (r)	No. of families (n)	P of r or more matches out of n
1–35	33	35	<.00005*
1–30	28	30	<.0004*
31–35	5	5	<.001†

* The inclusion of siblings in families 1, 10, 27, and 30 did not affect the calculation. Inclusion of the sibling in family 14 had a negligible effect of the P value.
† Calculated from the frequencies of variants 1, 16, and 27 on the basis of data in figure 1.

as in studies of HBV infections that are possibly related to blood transfusion, transplantation, sexual activity, needle sticks, and so forth.

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