Vol. 195, No. 1, 1993
August 31, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 186-191

Unique PreS Sequence in a Gibbon-Derived Hepatitis B Virus Variant

Larry T. Mimms**, Larry R. Solomon, James W. Ebert* and Howard Fields*

Experimental Biology
Abbott Laboratories
North Chicago, IL 60064

*Centers for Disease Control
Atlanta, GA 30333

Received July 14, 1993

A unique hepatitis B virus (HBV) variant has been identified in a gibbon (Hylobates lar) which could be passed to a chimpanzee by experimental inoculation. This HBV variant had been shown to have no reactivity to a monoclonal anti-preS2 antibody (preS2 mAb 116-34) differentiating it from all human HBV specimens tested. This gibbon sera also was not recognized by an anti-preS1 mAb which binds the preS1 hepatocyte receptor region, amino acids 27-35. In this paper, we report the DNA sequence of the gibbon HBV PreS gene. The lack of preS2 mAb (116-34) binding can be explained by a unique nucleotide substitution of A for C in the second codon of the preS2 region leading to the replacement of glutamine with lysine. Two other unique changes were observed at the seventh and 24th amino acid positions in the preS2 gene leading to a substitution of a valine for threonine and alanine, respectively. Unlike all human derived HBV sequences in the preS1 region, the gibbon HBV had a glutamic acid instead of an aspartic acid at amino acid residue 27. Another unique substitution was a leucine for alanine at preS1 position 33. These amino acid changes in the gibbon HBV may explain its unique preS mAb reactivity.
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The envelope of the hepatitis B virus (HBV) consists of three proteins and their glycosylated forms (3). The major HBV envelope protein is encoded by the S gene and exists as both glycosylated (p27) and unglycosylated forms (p24). The preS region contains two initiation codons in frame with the S gene allowing the expression of the M protein (55 amino acids of preS2+ S) and L protein (108 or 119 amino acids of preS1+preS2+S). M protein consists of singly and doubly glycosylated forms, gp33 and gp36, respectively. L protein has an unglycosylated (p39) and a singly

** To whom correspondence should be addressed.

0006-291X/93 $4.00
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glycosylated form (gp42). We have previously shown that preS monoclonal antibodies (mAb) can be used to detect antigenic variability among HBV subtypes (4). In another study, we identified a unique HBV variant in a gibbon (Hylobates lar) which was experimentally passaged to a chimpanzee (1,2). This variant showed no reactivity to a preS2 mAb (116-34) thus differentiating the gibbon HBV from all human HBV specimens tested. In this study we have determined the DNA sequence of the preS1 and preS2 regions of the gibbon HBV genome.

Materials and Methods

Experimental Animals

The HBsAg seropositive, white-handed gibbon (Hylobates lar) reported in these studies was imported into the US from Thailand. The animal had apparently been infected in the wild and was housed at the CDC from October 1987 to November 1988. The gibbon remained HBsAg positive for over two years and was classified as an HBV carrier (2). Although the animal was repeatedly negative for anti-HBc, immunofluorescent staining revealed the presence of HBcAg in liver biopsy specimens. A chimpanzee, Hope(CH1357), was inoculated i.v. with 1 mL of the gibbon sera. In this chimpanzee, HBsAg was detected at day 14 post inoculation, peaked at day 38 and persisted to day 73. A single marginally elevated ALT was found on day 93 post inoculation. Anti-HBc was detected beginning at day 112 and surprisingly occured 49 days after seroconversion to anti-HBs.

Immunoassays

HBsAg, anti-HBs and anti-HBc were measured using commercial EIA or RIA kits (Abbott Laboratories, Abbott Park, IL.). For HBV subtyping, mAb against the HBV preS region were produced, characterized and radioiodinated. Radioimmunoassays were conducted as descibed previously (4).

HBV DNA isolation, cloning and sequencing

HBV was captured from the Chimpanzee sera using a modification of the microparticle capture procedure described by Zeldis et al (5). Briefly, three separate microparticle preparations coated with either two monoclonal antibodies against preS1; two monoclonal antibodies against preS2; or a single monoclonal antibody against the 'a' epitope of HBV surface antigen were mixed in equal proportions at a final concentration of 0.5% (w/v) solids in 150 mMTtris (pH 8.0), 100 mM NaC1, 0.5% gelatin, 0.1% Tween-20, 9.5% sucrose and 0.02% NaN3. A 250 µl aliquot of chimpanzee sera was diluted to 500 µl with PBS and incubated with 125 µl of the microparticle mixture at room temperature for 30-60 min. The particle/HBV complex was recovered by centrifugation, washed with PBS, resuspended in 100 µl water and heat-treated at 95 C for 10 min. The mixture was digested with Proteinase K for 1 hour at 60 C after addition of an equal volume of 20 mM Tris-HC1 (pH 8.3), 100 mM KC1 and 200 µg/ml proteinase (Sigma Chemical Corp., St. Louis, MO). The proteinase K was inactivated by incubation at 95 C for 10 min. and the particles were removed by centrifugation at 12,000 x g for 10 min. PCR amplification was performed by mixing 40 µl of Proteinase K treated sample and 60 µl of PCR master mix for a final PCR reaction mixture of 10 mM Tris-HC1, pH 8.3: 50 mM KC1; 3 mM MgC12; 0.2 mM each dATP, dCTP, dGTP and TTP; 0.5 um primer P-2814F (5'-Pi-CTCTGCAGGATCCGG-

GTCACCATATTCTTGG-3'); 0.5 uM primer P-843R (5'-TACTGCAGTGAGGGTTT-AAATGTATACCC-3') and 25 U/ml AmpliTaq DNA polymerase (Perkin Elmer-Cetus Corp., Norwalk, CT). The mixture was heated to 95 C for 20 min. and amplified for 40 cycles of 94 C, 30 sec. 60 C, 30 sec. and 72 C, 45 sec. in a Perkin Elmer GeneAmp PCR system 9600. Following a 4 min. extension at 72 C, the PCR products were purified using GlassMax DNA purification system (Gibco/BRL, Bethesda, MD) according to the manufacturer's recommendations. Purified DNA was made blunt-ended using T4 DNA polymerase (Boerhinger Mannheim, Indianapolis, IN) followed by the Klenow fragment of E. coli DNA polymerase I (GIBCO/BRL) using standard procedures (6). The DNA was recovered by ethanol precipitation and ligated to phosphorylated HindIII linkers (Promega Corp., Madison, WI) according to the manufacturers recommendations. The ligation product was digested with HindIII (Promega) after which the 1.2 kb full-length product was purified from low melting agarose with Magic PCR preps DNA purification system (Promega) and subcloned into HindIII digested/dephosphorylated plasmid pSP72 (Promega) using T4 DNA ligase (Promega). 3-5 µl of the ligation reaction was used directly to transform competent E. coli DH5A cells (GIBCO/BRL) to ampicillin resistance according to the manufacturers recommendations. Plasmid DNA was purified from positive colonies using Magic Maxipreps DNA purification system according to the manufacturer's recommendations (Promega). Both strands of the preS region were completely sequenced using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Cleveland, OH) and the following primers: T7 P/P (5'-TAATACGACTCACTATAGGG-3') or SP6 P/P (5'-ATTTAGGTGACACTATAG-3'), 3013F (5'-AACAAGGTGGGAGTGGGAGC), 3178 F (5'-ACCTCTAAGGGACACTCATC-3'), 251R (5'-AGACTCTGTGGTATTGTGAG-3'), 004R (5'-GGAGTTCCACTGCATGGCCT-3') and 3090R (5'-GCCTGAGGGCTCCACCCCAA-3'). The use of T7 P/P or SP6 P/P depended on the orientation of the insert as determined by restriction analysis. Primer designations represent the position of the 5' end of the oligonucleotide relative to the EcoRI site in strain adw.

The numbering of the preS proteins in the text and Tables is as described by Neurath et al (7). Residue 1 begins at the first initiation codon of the preS1 region of the adw2 or adr subtype. Eighteen complete HBV DNA sequences were obtained in Genebank and 17 HBV sequences not deposited in Genebank were obtained from Roger Miller at NIH (8). These sequences were aligned using the MacDNASIS program.

Results and Discussion

Sera from a gibbon chronically infected with HBV was tested for reactivity to anti-preS and S monoclonal antibodies. The gibbon HBV was unique in showing no reactivity to preS2 mAb group 2a(116-34) (Table 1). The gibbon HBV also gave little or no reactivity with preS1 group 1 mAb, 116-80, which had been shown to bind within the preS1 hepatocyte binding site at residues 27-35 (9). Gibbon sera was also unreactive with preS1 group 2 mAb 115-16, similar to the Paris subtypes ayw1,adw2 and ayr (4). All other preS and S mAb binding was similar to that of the Paris ayw2/ayw3 subtype. To determine whether this gibbon HBV strain was transmissable to other primates, the gibbon sera was inoculated into a chimpanzee. The

Table 1. Unique PreS binding to Gibbon (Hylobates lar) HBV

		Specimen		
Group	Clone number	Paris ayw2	Gibbon	Chimpanzee: Hope
S mAb				
	H10	+++	+++	+++
	H95	-	-	-
PreS2 mAb				
1	50-80	+++	+++	+++
	116-183	+++	+++	+++
	25-19	+++	+++	+++
	128-410	+++	+++	+++
2a	116-34	+++	-	-
2b	115-32	+++	+++	+++
3	128-603	+++	+++	+++
PreS1 mAb				
1	116-80	+	-	-
2	116-86	++	++	++
	115-16	++	-	-

+++ represents S/N >100, ++ represents 20-100, + represents 5-20, and - represents S/N <5. Taken from reference (1).

chimpanzee subsequently developed a serologically confirmed hepatitis B infection. Serum from the gibbon and chimpanzee had an identical pattern of S and preS mAb binding (Table1).

The preS gene of the acutely infected chimpanzee was PCR amplified, cloned and sequenced. Two clones from the chimpanzee sera were derived from independent PCR reactions and both had identical preS nucleotide sequences. There were 5 unique nucleotide substitutions in the amino terminal half of the preS2 gene compared to the 35 published HBV sequences (8). These substitutions led to 3 unique amino acid changes (Table 2). The nucleotide substitution of A for C in the second codon of the preS2 region lead to the replacement of glutamine with lysine. This substitution likely explains the lack of preS2 mAb (116-34) reactivity since this mAb has been shown to bind at or near the N terminus of the M protein. The binding of 116-34 depends on glycosylation of the asparagine at residue 123 since treatment of M protein with endoglycosidase F completely destroys binding of this mAb (4). Carbohydrate may be part of the antibody binding site or alternatively may affect the polypeptide conformation. It is not known whether the mutation prevents glycosylation

TABLE 2. Amino acid sequence of PreS2 region (120-152) derived from the published nucleotide sequence for various HBV subtypes*

HBV Id	Reference	120										130										140										150		
		M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	A	G	G	S	S	S	G	T	V
ADR	(12)																																	
ADYW	(13)		H										T	Q											A									
BLUM	(14)							A						Q																				
INDO	(15)												T	Q																				
Liang	(Liang et al, unpublished)		H			Y							T	Q																				
ADRC	(16)																	V																
ADRCG	(17)												V												P									
Chimp	(18)													Q																				
Hope	This work		[K]					[V]				T		Q											[V]									

*Only HBV sequences having unique amino acid changes in the N terminal half of PreS2 are shown.
- Indicates no amino acid change from the ADR sequence. Boxed amino acids indicate unique amino acid substitutions in this work.

or directly disturbs the epitope. Two other unique changes were observed at the seventh and 24th amino acid positions in the preS2 gene leading to a substitution of a valine for threonine/alanine. These substitutions would be expected to have little effect on 116-34 binding compared to the glutamine to lysine substitution at position 121.

Unlike all published human derived HBV sequences in the preS1 region, the gibbon HBV had a glutamic acid instead of an aspartic acid at amino acid residue 27 and a leucine for alanine substitution at preS1 position 33 (Table 3). Interestingly, the only chimpanzee HBV sequence in Genebank also had a glutamic acid at residue 27. It is possible that one or both of these amino acid changes may affect anti-preS1 mAb (116-80) binding since the binding site for this mAb has been mapped to residues 27-35. This region of preS1 is also within the preS1 hepatocyte receptor (7,10). The Paris subtype adw4 also showed weak reactivity to this mAb however the preS1 sequence of this subtype has not been determined (4). An alternative explanation for the poor mAb 116-80 reactivity may be the presence of factors in the sera which bind to the preS1 hepatocyte receptor region blocking mAb binding.

TABLE 3. Amino acid sequence of PreS1 region (12-50) derived from the published nucleotide sequence for various HBV subtypes*

HBV Id	Reference	12							20								30								40									50
		M	G	T	N	L	S	V	P	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	G	A	N	S	N	N	P	D	W	D	F	N	P	N	K	D
ADR	(12)																																							
ADYW	(13)																								R			T												
LIANG	(Liang et al, unpublished)			Q			T	S																	R			T	A										
MAY4	(11)			Q			T	S																	R			T	R										
ADW	(12)			Q			T	S							L														T								I		
ADW1	(11)																								K				E								H		
ADW2	(11)																								K				E										
ADWZ	(15)																								K				D								H		
HEPB	(GenBank X51970)																						V														I		
Chimp	(18)			Q			T	S									[E]								K			T									K		
Hope	This work			Q				S									[E]						[L]		K														

*Only HBV sequences having unique amino acid changes in the N terminal half of PreS1 are shown.
- Indicates no amino acid change from the ADR sequence.

Overall the gibbon HBV PreS sequences were similar to published sequences suggesting that the gibbon HBV would not represent a distinct group from those described by Okamoto et al.(11) and Ogata et al. (8). HBV group classification of the gibbon HBV awaits sequencing of the entire genome.

Acknowledgment

Thanks to Roger Miller of the National Institutes of Health for providing HBV sequences not currently entered into GenBank.

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