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Selection pressure on the hepatitis B virus pre-S/S and P open reading frames in Tongan subjects with a chronic hepatitis B virus infection

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ABSTRACT

Identification of the full repertoire of hepatitis B virus (HBV) peptides that are presented to CD8+ T cells by common HLA class I alleles will be useful for designing immunotherapies for chronic hepatitis B. One hundred and seventy five cloned sequences containing the pre-S/S and P open reading frames (ORF) of the HBV were obtained from serum HBV-DNA of HBeAg-positive ($n = 4$) and HBeAg-negative (inactive healthy carriers (IHC), $n = 16$) Tongan subjects with an inactive chronic HBV infection. In addition, 34 and 32 sequences were obtained 5.2 ± 1.4 (mean ± SD) years apart from eight subjects. PAML was used to identify codons in the pre-S/S and P ORFs that were under positive selection pressure ($\omega > 1$). The number of non-synonymous substitutions in these codons was compared in IHC who were homozygous for either HLA-B*4001 ($n = 9$) or HLA-B*5602 ($n = 7$), and who were either positive ($n = 6$) or negative ($n = 10$) for HLA-A*02. 34 codons in the pre-S/S and 11 codons in the P ORFs were under positive selection pressure. There was a higher number of non-synonymous substitutions in these codons in HBeAg-negative versus HBeAg-positive subjects in the P ($p = 0.02$) but not the pre-S/S ($p = 0.64$) ORF. There was no association between any HLA class I allele and non-synonymous substitutions in these codons. There was no increase in positive selection pressure on the pre-S/S and P ORFs with time. In conclusion, we could not find HLA class I-restricted selection pressure on any pre-S/S or P ORF amino acid; raising the possibility that peptide-based immunotherapies for chronic hepatitis B may not require peptides from these ORFs.

1. Introduction

Chronic hepatitis B virus (HBV) infections usually evolve through three stages (Hoofnagle et al., 2007; Lok and McMahon, 2007). Initially, the virus avoids eradication by the host immune system using mechanisms that are not fully understood. Possibilities include suppression of the host immune system by the viral HBeAg (Milich and Liang, 2003), and high levels of viral antigens causing T cell exhaustion (Maini et al., 2000; Reignat et al., 2002). This results in high levels of viral replication in the liver, high levels of serum HBsAg and HBeAg, and no detectable liver inflammation. This is the 'immune tolerant' stage of the disease. Immune tolerance to the HBV is eventually lost in most patients, for reasons that are unknown. Immunity to HBV antigens becomes detectable in peripheral blood mononuclear cells, serum levels of HBV-DNA and HBeAg fall, anti-HBe antibodies appear in serum, and there is usually evidence of liver inflammation. This process is known as HBeAg seroconversion, and it eventually leads to CD8+ T cell-mediated suppression of viral replication, clearance of the HBeAg from serum, and liver inflammation ceases. This is the inactive, healthy carrier (IHC) stage. HBsAg may also disappear in a small number of subjects, associated with the appearance of anti-HBs antibodies.

There is a subset of patients who do not suppress viral replication at the time immune tolerance is lost. These patients, who can be either HBeAg-positive or HBeAg-negative, develop persistent liver inflammation that is known as chronic hepatitis B (CHB). CHB is associated with a high risk of liver cirrhosis and hepatocellular

Abbreviations: CHB, chronic hepatitis B; IHC, inactive healthy carrier; ORF, open reading frame.

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carcinoma (Lok and McMahon, 2007). Lifelong treatment with anti-viral drugs that suppress replication of the virus is currently required to prevent these sequelae (Lok and McMahon, 2007; Reijnders et al., 2010). These treatments are expensive, require frequent monitoring and can be associated with life-threatening flares of hepatitis (Lim et al., 2002; Lok and McMahon, 2007). A therapeutic vaccine that stimulated the patient's own immune system to permanently suppress viral replication would make a valuable contribution to management of CHB.

Randomised, controlled trials of therapeutic vaccines in CHB using pre-S/S open reading frame (ORF) antigens have shown modest (Hoa et al., 2009), if any (Vandepapeliere et al., 2007), increases in efficacy relative to Lamivudine treatment (Horiike et al., 2005; Michel et al., 2011). The reasons for the poor clinical responses include exhaustion or deletion of CD8+ T cells that respond to S peptides (Michel et al., 2011; Reignat et al., 2002; Webster et al., 2004). The minimum criterion for a successful response to a therapeutic vaccine is the permanent development of the IHC state, as this is associated with a significant decrease in risk for liver cancer (Lok and McMahon, 2007). There is currently no evidence that immune responses to any pre-S/S and P open reading frame (ORF) antigens contribute to the suppression of HBV replication that occurs in IHCs (Martinet et al., 2012; Webster et al., 2004), and evolutionary rate analyses show low rates of pre-S/S and P relative to C ORF evolution (Harrison et al., 2011). These findings suggest that it will be difficult to induce immune responses to pre-S/S and P ORF epitopes with therapeutic vaccines. This is in contrast to immunological (Maini et al., 2000; Webster et al., 2004) studies which show evidence of functional, HBV core peptide-specific CD8+ T cell responses in IHCs. Phylogenetic studies also provide evidence that the immune response to the core gene contributes to the suppression of HBV replication in IHCs. There is a higher frequency of positively-selected, core gene escape substitutions in the IHC than in either the CHB or the immune tolerant stages of the disease (Abbott et al., 2010; Warner et al., 2011), these substitutions evolve over time within-patients (Warner et al., 2011), and some of these substitutions are HLA class I restricted (Abbott et al., 2010). These three observations would be expected if CD8+ T cells suppressed viral replication in IHC.

Although CD8+ T cells that recognise epitopes in the HBV pre-S/S and P ORFs exist in some IHC patients (Webster et al., 2004), there is still a need for data to demonstrate that they contribute to suppression of replication. Thus the goals of this study were to determine whether positive selection pressure on any pre-S/S and P ORF codons is HLA class I-restricted, whether there is a higher frequency of substitutions in these positively-selected codons in HBeAg-negative relative to HBeAg-positive subjects, and whether the frequency of these substitutions increases with time using within-subject longitudinal data. This study was carried out in the Tongan population that are resident in Auckland, New Zealand. There is a high prevalence of chronic HBV infection in Tonga, due to a combination of both horizontal and vertical transmission (Wainwright et al., 1986).

2. Subjects and methods

2.1. Patients and blood samples

The New Zealand Hepatitis B Screening Programme monitors 1,439 Tongan adults with a chronic HBV infection at 6–12 monthly intervals. We serially recruited 345 of these subjects into this study at the time of a routine blood test. An extra 3 ml of serum was taken for HBV-DNA analyses, and genomic DNA was extracted from peripheral blood leucocytes for HLA class I genotyping. All subjects gave written consent, and the study was approved by the Northern X Regional Ethics Committee of the New Zealand Ministry of Health.

Initially, the full HBV genome from the serum of 47 of the 345 subjects was sequenced for the purpose of determining the wild-type sequences of the HBV in Tonga, and identifying conserved primer sites for sequencing and PCR. Next, all IHC subjects with a genotype C infection, who had PCR detectable HBV DNA, and who were homozygous for either the HLA-B*4001 allele ($n = 11$) or the HLA-B*5602 allele ($n = 9$) were selected for a cross-sectional analysis of pre-S/S and P ORF substitutions. This group comprised 20 of the 21 subjects described in a similar study of HBV core gene substitutions (Abbott et al., 2010). None of these subjects had ever been treated for chronic hepatitis B. The age (mean ± s.e.m), gender, serum ALT (mean ± s.e.m) and HBV DNA levels (median (range)) and HBeAg status of the groups at the time of the analysed serum sample are shown in Table 1; as are the number of subjects with each identified HLA-A and HLA-C allele. The G1896A precore mutation was present in one of four HBeAg-positive subjects and 13 of 16 HBeAg-negative subjects. The A1762T core promoter mutation was present in one of four HBeAg-positive subjects and eight of 16 HBeAg-negative subjects. The G1764A/T core promoter mutation was present in one of four HBeAg-positive subjects and 11 of 16 HBeAg-negative subjects.

One HBeAg-negative subject (subject N1 in Supplementary Tables 1–3) with an HBV-DNA of 5.3 $\log_{10}$ IU/ml had a liver biopsy 34 months after the analysed serum sample for assessment of a flare of hepatitis. This biopsy was consistent with chronic hepatitis B, grade 2, stage 2.

A separate group of eight immune tolerant subjects was then selected for a longitudinal study in which selection pressure on the HBV C, pre-S/S and P ORFs was compared on virus samples obtained on two occasions from each subject. Five of these subjects, aged 44.6 ± 6.0 (mean ± S.D.) years at baseline, underwent spontaneous HBeAg seroconversion before giving a follow up serum sample at 5.7 ± 0.3 (mean ± S.D.) years after the baseline sample. Three of these subjects, aged 25.4 ± 4.3 (mean ± S.D.) years at baseline, remained HBeAg-positive for 4.4 ± 1.1 (mean ± S.D.) years before their follow up sample.

Table 1
Description of the subjects with an inactive chronic hepatitis B virus infection who were homozygous for either HLA-B*4001 or HLA-B*5602. The number of subjects with each HLA-A and HLA-C allele are shown, with the number of homozygotes in parenthesis.

	HLA-B*4001	HLA-B*5602
Age (years)	47.5 ± 4.2	46.8 ± 4.1
Gender (male/female)	7/4	5/4
ALT (normal < 45 U/L)	32.9 ± 6.7	40.9 ± 15.3[b]
HBeAg (positive/negative)	2/9	2/7
[HBV DNA] HBeAg +ve ($\log_{10}$ IU/ml)	2.8 and >8.2	5.8 and >8.2
[HBV DNA] HBeAg –ve (median (range) $\log_{10}$ IU/ml)	1.9 (<1.3–3.6)[c]	2.2 (<1.3–3.8)
HLA-A alleles		
*02[a]	7 (2)	0 (0)
*1101	1 (0)	3 (0)
*2402	7 (3)	9 (4)
*2601	1 (0)	0 (0)
*3401	1 (0)	2 (0)
HLA-C alleles		
*0102	0 (0)	9 (8)
*0304	10 (5)	0 (0)
*0401	6 (1)	0 (0)
*1203	0 (0)	1 (0)

[a] HLA-A*0201 ($n = 5$), *0206 ($n = 4$).
[b] ALT = 153 U/L in one subject with NAFLD.
[c] This summary excludes one subject with HBV-DNA = 5.3 $\log_{10}$ IU/ml (see text).

2.2. Cloning and sequencing the HBV

This study describes the results of cloning and sequencing a 2.6 kb amplimer from the HBV that included the entire pre-S/S ORF, the terminal 2,455 bp of the P ORF and the initial 403 bp of the X ORF. The rationale for choosing the PCR primers, the PCR conditions and the cloning and sequencing strategies have been described in detail in a previous publication (Abbott et al., 2010). The average number of 2.6 kb clones per patient was 8.8 ± 1.9 (mean ± S.D.) with a range of 5–12. The presence of indels and/or nonsense mutations reduced the number of clones from some patients that were suitable for some analyses. The frequencies of canonical mutations in the precore region and basal core promoter were obtained from analysis of previously published clones containing the HBV C open reading frame (Abbott et al., 2010).

2.3. Serum HBV-DNA levels

HBV-DNA levels were measured using the COBAS Ampliprep/COBAS Taqman HBV Test, version 2.0, which has a linear range of 1.30–8.23 $\log_{10}$ IU/ml.

2.4. Genotyping the HBV

The HBV from each subject was genotyped as previously described (Abbott et al., 2010).

2.5. Selection analyses of HBV clones

The nature of the selection pressures acting on the HBV viruses was investigated using the methods of Yang et al. (2005). In these methods, the rate at which amino-acid altering (non-synonymous) substitutions occur (*dN*) in the codon sequence is compared with the rate for non-altering (synonymous) substitutions (*dS*). These rates are usually compared by calculating their ratio (often represented as ω). When evolution occurs by the random processes of mutation and drift of nucleotides, we expect the rate of non-synonymous substitutions to be the same as that for synonymous substitutions ($\omega = 1$) and selection is said to be neutral. When amino acid-altering substitutions are disadvantageous, and purged, then the ratio ω will fall in the range 0–1. This represents negative selection. When selection favours amino acid diversity, the rate of non-synonymous substitutions exceeds the rate expected by random processes ($\omega > 1$), to give positive selection. These substitution rates of codon evolution must be inferred on a phylogenetic tree.

The selection analyses were performed in two ways. First, phylogenies of the viruses obtained from the different patients were constructed using the maximum likelihood method in PhyML (Guindon et al., 2004). PAML model 2a, (Yang et al., 2005) was then used to estimate the overall proportion of codons in the whole alignment that were under positive, neutral or negative selection pressures across the whole tree. Each codon was then assigned to the selection category (negative, neutral or positive) to which it had the greatest posterior probability of belonging, as calculated using the Bayes Empirical Bayes (BEB) criterion. In the second method, the BEAST program (Drummond and Rambaut, 2007) was used first to estimate the phylogeny under a model of a constant evolution rate in longitudinal data. The samples were assigned to one of two groups based on time (early versus late samples). Then, PAML (model 2b) was used to identify differences in the positive selection pressure on HBV genes by estimating ω at one timepoint (foreground) relative to the second timepoint (background) in which ω was constrained to one.

2.6. General statistics

Numerical data are summarised as the mean ± the standard error of the mean unless otherwise stated. Ordinal data are summarised as the median (range). Comparisons of ordinal data between groups were conducted with a Kruskal–Wallis Test. Comparisons of frequency data between groups were conducted with a Fisher's exact test. Statistical calculations were performed using SAS (SAS Institute Inc. Cary, NC).

3. Results

3.1. Sequence of wild-type, sub-genotype C3 hepatitis B virus in Tonga

In order to correctly identify mutations in the HBV-DNA from individual Tongan subjects, it is necessary to obtain a consensus sequence for the wild type, Tongan, sub-genotype C3 HBV (Norder et al., 2004). The full length HBV genome was sequenced from the serum of three samples of Tongan subjects, sequentially taken from our cohort of 345 subjects with a chronic HBV infection. The first two groups comprised 11 (bold branches and bold type in Fig. 1) and 20 (ID begins with P and normal type in Fig. 1) subjects with an immune tolerant chronic HBV infection. The third group comprised 16 IHC subjects (ID begins with N in Fig. 1). Phylogenetic analyses identified two sub-groups of C3 HBV in each of the three samples, which could be distinguished by the same haplotype of 65 nucleotides. Obtaining this result in all three patient samples shows that there are two sub-sub-genotypes of the C3 sub-genotype HBV in the Tongan population, and excludes the possibility that stochastic population genetic processes were responsible for the finding. Thus it was necessary to define two consensus sequences for the C3 sub-genotype in Tongan subjects. These consensus sequences were defined as the most common nucleotide at each position in the 15 sub-sub-genotype C3A and the 12 sub-sub-genotype C3B subjects in Fig. 1 who were HBeAg-positive.

3.2. Phylogenetic analysis of 2.6kb Clones

Fig. 2 shows the phylogenetic tree assembled from 175 sequences of HBV-DNA clones extracted from the 20 subjects in Table 1. Each clone contained the 2,611 bp sequence running from bases 2,381–1,776 (GenBankID: X75656). Thirty-five of the clones came from four HBeAg-positive subjects (blue in Fig. 2), and 140 of the clones came from 16 HBeAg-negative subjects (red vertical branches). The number of clones from each subject was adequate to define the repertoire of fixed or almost fixed nucleotide substitutions in the HBV from that subject. These substitutions define a unique HBV haplotype in each subject, and demonstrate that there was no cross-contamination of clones between patient samples. The fixed and almost-fixed substitutions include the nucleotides that are under the strongest selection pressure (Locarnini et al., 2003). These are likely to be of most value in the search for immune epitopes that will be useful in a therapeutic vaccine.

3.3. Analyses of selection pressure on the HBV S gene

3.3.1. S codons under selection pressure

The S gene sequences from 171 of the 175 clones were analysed in PAML. Two clones from each of subjects P1 and N16 were removed because of indels. Maximum likelihood analysis estimated that 26.8% of the 226 codons in the S gene were under negative selection ($\omega = 0.00$), 63.0% were under neutral selection ($\omega = 1.00$), and 10.2% were under positive selection ($\omega = 5.99$). Site by site analysis identified 19 codons under positive selection ($\omega > 1.0$). The level of positive selection pressure on 11 of these

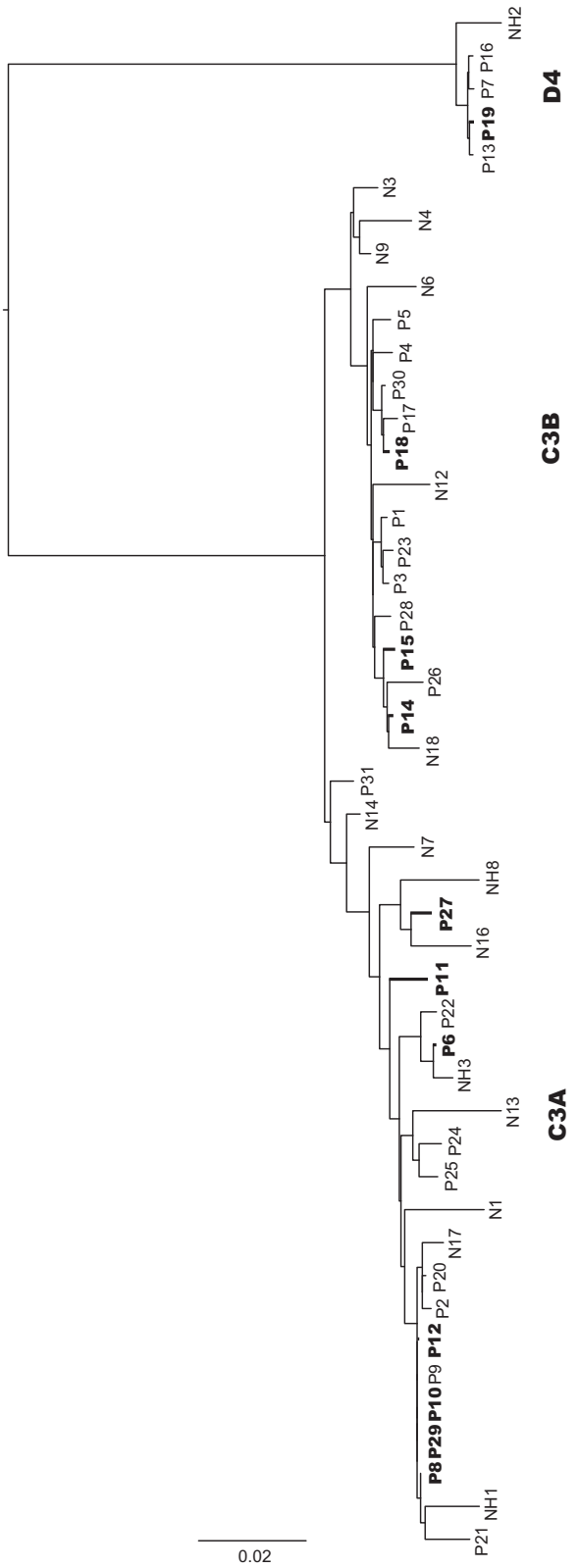

Fig. 1. Phylogenetic tree constructed from 47 complete HBV-DNA sequences extracted from the serum of Tongan subjects with either an HBeAg-positive (ID prefixed with a P, $n = 31$) or HBeAg-negative (ID pre-fixed with an N, $n = 16$) chronic HBV infection. A bold **P** indicates the first group of subjects sequenced. A normal P and N indicates the second and third groups of subjects sequenced respectively. The two sub-sub-genotypes of the genotype C hepatitis B virus, named C3A and C3B, are shown.

19 codons reached statistical significance (Table 2 and Fig. 3). The level of positive selection pressure on eight of these 19 codons did not reach statistical significance (Table 3). The coding for the S gene is entirely contained within the coding for the polymerase gene, although in a different reading frame. Thus there is potential for S gene substitutions to be caused by positive selection pressure on either one of the two polymerase codons that overlap the codon for each S amino acid. Although there was some positive selection on the polymerase gene (see below), this only occurred at codons outside of the overlap with the S gene. Thus none of the selection pressure on the S gene was the result of substitutions that occurred as a result of selection pressure on the polymerase gene.

3.3.2. Influence of HLA class I on selection pressure on the S gene

The numbers of clones with a non-synonymous substitution at each of the positively-selected codons in each subject are shown in Supplementary Table 1. Subjects N1–N10 were homozygous for HLA-B*4001 and subjects N11–N17 were homozygous for HLA-B*5602. There were no significant associations between HLA-B genotype and the frequency of substitutions at any positively-selected codon. There were no significant associations between non-synonymous substitutions at any codon and the presence (subjects N1–N7) or absence (subjects N8–N17) of an HLA-A*02 family allele.

We also used an algorithm (www.immuneepitope.org) to predict peptides in the S gene that might be presented by these HLA class I alleles to CD8+ T cells. The purpose of identifying these peptides was to determine whether they contained substitutions that had arisen at different amino acids in different subjects in order to escape Tcr recognition of the peptide. We selected peptides with a total score for proteosomal cleavage, TAP transport and HLA binding of greater than −1.0 and an IC50[nM] for HLA-binding of less than 500. Two peptides (aa 175–183 and aa 337–347) within the S gene fulfilled these criteria for presentation by HLA-B*4001. Amino acid substitutions were found in these regions in 3 and 0 of the 11 HLA-B*4001 subjects respectively; but this was no higher than the number of substitutions found in these regions in the 9 HLA-B*5602 subjects (1 and 1 subjects, respectively). Five regions (aa 300–308, aa 324–332, aa 351–360, aa 370–379, aa 384–396) fulfilled the criteria for containing a peptide presented by HLA-B*5602. Amino acid substitutions were found in these regions in 1, 1, 1, 3 and 3 of the 9 HLA-B*5602 subjects respectively; but this was no higher than the number of substitutions found in these regions in the 11 HLA-B*4001 subjects (4, 1, 3, 4 and 3 subjects respectively). Eighteen regions in the S gene fulfilled the criteria for containing an HLA-A*02 binding peptide, and there were 82 potential HLA-A*02 binding peptides within these regions. These included all the HLA-A*02 binding peptides previously identified by Nayersina et al. (1993). Although these 82 peptides contained 18 of the 19 positively selected amino acids within the S gene, the frequency of HLA-A*02-positive subjects with substitutions within these regions was similar to the frequency in the HLA-A*02-negative subjects (data not shown).

3.3.3. Influence of HBeAg status on S gene selection pressure

The median number of positively-selected codons in the S gene with at least one non-synonymous substitution in each subject was similar in the 16 HBeAg-negative (ID prefixed with N) and in the four HBeAg-positive (ID prefixed with P) subjects (Supplementary Table 1). For example, subject N1 had non-synonymous substitutions at 5 of these 19 codons (192V, 205S, 221V/R, 270 V, 378S) and subject P1 had non-synonymous substitutions at 8 of these 19 codons. This applied when the 11 codons under statistically-significant selection pressure in Table 2 were analysed (2 (0–7)

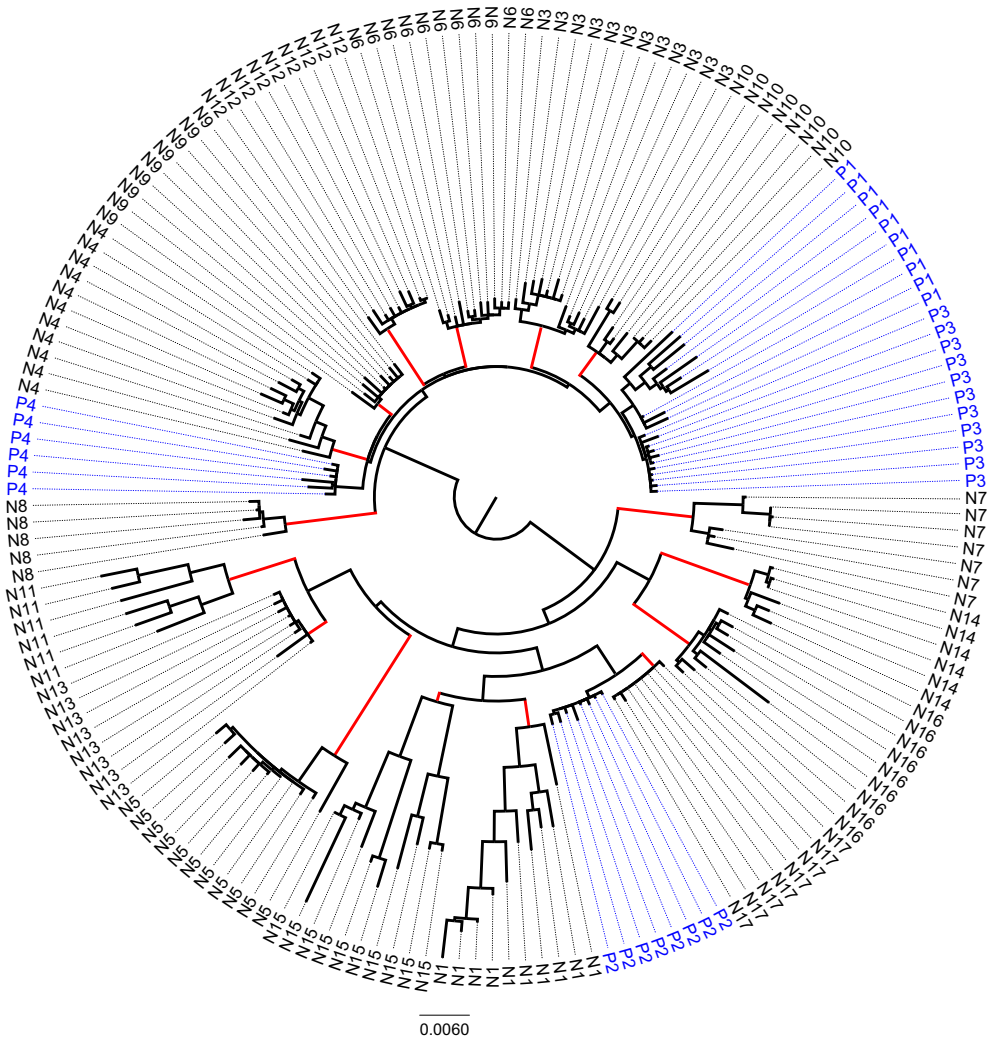

Fig. 2. Phylogeny of 175 clones containing 2,611 bp of genetic material from the hepatitis B virus extracted from the serum of 20 subjects with an inactive chronic HBV infection. Data from the four HBeAg-positive subjects are shown in blue. Each red vertical branch leads to a clade of clones that came from one HBeAg-negative subject. This figure demonstrates that there was no cross-contamination of clones between patient samples.

Table 2
Codons in the HBV S gene that were under statistically-significant, positive selection pressure. Codons are numbered by their position in the pre-S/S ORF/position in the S gene.

Codon	Wild type amino acid	Posterior probability	Omega (±s.e.m.)	Subjects +ve at each site[a]	
				HBeAg +ve	HBeAg −ve
184/10	G	1.000	5.7 ± 1.1	1	4
188/14	V	0.999	5.7 ± 1.5	1	4
192/18	V	0.963	5.5 ± 1.4	1	2
221/47	V/R[b]	0.976	5.6 ± 1.3	1	2
270/96	V	0.990	5.6 ± 1.2	1	4
275/101	Q	0.995	5.7 ± 1.2	0	4
334/160	R	0.998	5.7 ± 1.2	3	1
358/184	A/V[b]	0.992	5.7 ± 1.2	1	4
378/204	S	0.995	5.7 ± 1.2	1	4
384/210	S	0.971	5.5 ± 1.4	0	2
391/217	P	1.000	5.7 ± 1.1	0	5

[a] The number of subjects with at least one clone with a non-synonymous substitution.
[b] The wild type amino acid in sub-sub-genotypes C3A and C3B, respectively.

versus 1.5 (1–6) codons with a non-synonymous substitution in HBeAg-negative versus positive groups respectively, $p = 0.92$) and when all 19 codons in Tables 2 and 3 with an $\omega > 1.0$ were analysed (3 (0–10) versus 1.5 (1–8)) codons with a non-synonymous substitution in HBeAg-negative versus-positive groups respectively, $p = 0.63$).

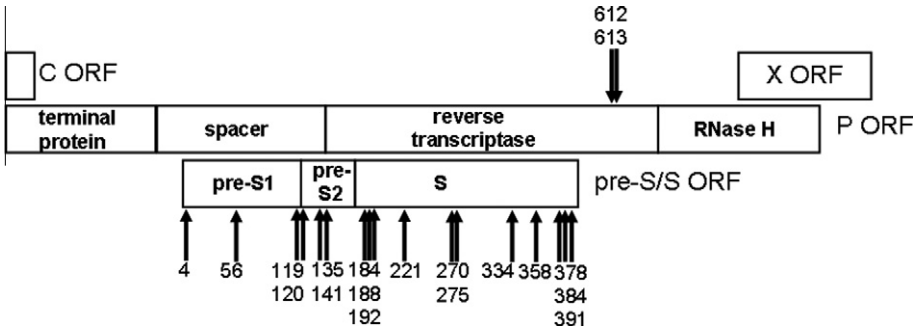

Fig. 3. The position of codons in the P and pre-S/S open reading frames (ORF) that were under statistically significant (posterior probability >0.95), positive selection pressure. Numbering is from the beginning of the P and pre-S/S ORFs.

Table 3
Codons in the HBV S gene that were under positive selection pressure ($\omega > 1.0$), but did not reach statistical significance. Codons are numbered by their position in the pre-S/S ORF/position in the S gene.

Codon	Wild type amino acid	Posterior probability	Omega (±s.e.m.)	Subjects +ve at each site[a]	
				HBeAg +ve	HBeAg −ve
176/2	E	0.906	5.2 ± 1.7	0	1
205/31	S	0.526	3.3 ± 2.3	1	2
213/39	L	0.513	3.2 ± 2.2	0	3
214/40	N	0.904	5.2 ± 1.7	0	3
217/43	G	0.736	4.4 ± 2.2	1	1
219/45	A	0.511	3.2 ± 2.3	0	3
300/126	I	0.906	5.2 ± 1.7	0	5
363/189	T	0.750	4.4 ± 2.2	0	2

[a] The number of subjects with at least one clone with a non-synonymous substitution.

3.4. Analyses of selection pressure on the pre-S1 and pre-S2 sequences

The full pre-S/S open reading frame from 156 of the 175 clones was analysed in PAML. Nineteen clones from eight subjects were removed because they contained indels and/or nonsense mutations. Maximum likelihood analysis estimated that 34.5% of the 400 codons in the full pre-S/S ORF were under negative selection ($\omega = 0.12$), 58.3% were under neutral selection ($\omega = 1.00$), and 7.3% were under positive selection ($\omega = 4.9$). Site by site analysis identified 15 codons in the pre-S1 and pre-S2 sequences under positive selection ($\omega > 1.0$). Selection pressure on 6 of these 15 codons reached statistical significance (Table 4 and Fig. 3). The coding for pre-S genes is contained within the coding for the polymerase gene, and statistically-significant, positive selection pressure on four codons in the pre-S region was associated with non-significant selection pressure on an overlapping codon in the P open reading frame (‡ in Table 4). This suggests that the P ORF substitutions at these sites are secondary to selection pressure on the pre-S region. Non-significant selection pressure on the codon for pre-S amino acid 55A ($p = 0.895$) was associated with non-significant selection pressure on the overlapping codon for amino acid 235P ($p = 0.587$) in the P ORF (Table 4).

HBeAg status did not influence the frequency of positively-selected codons with non-synonymous substitutions in either the pre-S region or the entire pre-S/S ORF respectively when either the statistically significant codons were analysed ($p = 0.10$, $p = 0.60$) or when the codons with $\omega > 1.0$ were analysed ($p = 0.14$, $p = 0.64$). There were no differences in the number of non-synonymous substitutions at any of the 15 codons with $\omega > 1.0$ between the groups with and without either the HLA-B∗4001, HLA-B∗5602 or HLA-A∗02 alleles (Supplementary Table 2).

We also used the www.immuneepitope.org algorithm to predict peptides in the pre-S region that might be presented by these HLA class I alleles to CD8+ T cells. There were no predicted HLA-B∗4001-binding peptides. There were four regions that contained potential HLA-B∗5602-binding peptides (aa 20–34, aa 104–112, aa 141–151 and aa 152–162). The number of subjects with amino acid substitutions within these four regions was similar in the HLA-B∗5602 (0, 0, 2, 1) and HLA-B∗4001 (0, 0, 3, 1) subjects respectively. There were six regions with potential HLA-A∗02-binding peptides in the pre-S region. Although three of these contained amino acids under positive selection pressure, the overall frequency of substitutions in these regions was similar in HLA-A∗02-positive and HLA-A∗02-negative subjects (data not shown).

3.5. Analyses of selection pressure on the P open reading frame

The coding sequence for the terminal 818 of the 843 codons in the polymerase gene was analysed from 155 of the 175 clones in PAML. Twenty clones from nine subjects were removed because they contained indels or nonsense mutations. Maximum likelihood analysis estimated that 79.6% of these 818 codons were under negative selection ($\omega = 0.13$), 19.8% were under neutral selection ($\omega = 1.00$), and 1.5% were under positive selection ($\omega = 3.77$). Site by site analysis identified 18 codons that were under positive selection ($\omega > 1.0$). The level of positive selection pressure on 2 of these 18 codons reached statistical significance. Only 5 of these 18 codons, including the two that were statistically significant, were in regions of the polymerase gene that do not overlap with other open reading frames (Table 5 and Fig. 3). Six of these 18 positively-selected codons were in regions that overlapped the C ($n = 1$), pre-S ($n = 3$) and X ($n = 2$) open reading frames, with no selection pressure on either of the overlapping codons (Table 5). Two of the 18 positively-selected codons (177Q and 180E) overlapped codons in the core gene (Abbott et al., 2010) and five overlapped codons in the pre-S region (Table 4) that are known to be under positive selection pressure. These seven codons are not included in Table 5.

Table 4
Codons in the pre-S1 and pre-S2 regions that were under positive selection pressure. A posterior probability of greater than 0.95 indicates statistical significance.

Codon	Wild type Amino Acid	Posterior Probability	Omega (±s.e.m.)	Subjects +ve at each site[a]	
				HBeAg +ve	HBeAg −ve
4[cP184]	W	1.000	4.9 ± 0.8	0	4
56[cP236]	N	0.997	4.9 ± 0.8	0	3
119	A	0.964	4.8 ± 1.1	0	4
120[cP301]	M	0.999	4.9 ± 0.8	1	7
135	R	0.992	4.9 ± 0.9	0	6
141[cP321]	F/Y[b]	0.981	4.8 ± 0.9	1	2
35	G	0.569	3.2 ± 2.0	0	1
51	Q/H[b]	0.731	3.8 ± 1.8	0	1
55[cP235]	A	0.895	4.5 ± 1.4	0	2
60	A	0.893	4.5 ± 1.4	0	3
84	I/T[b]	0.753	3.9 ± 1.8	0	4
132	L	0.794	4.1 ± 1.7	0	2
149	G	0.564	3.1 ± 2.0	1	1
158	A	0.530	3.0 ± 2.0	1	2
173	P	0.543	3.0 ± 2.0	0	2

[a] The number of subjects with at least one clone with a non-synonymous substitution.
[b] The wild type amino acid in sub-sub-genotypes C3A and C3B, respectively.
[c] Associated with selection pressure on an overlapping P ORF amino acid. Posterior probabilities: P184 = 0.871, P235 = 0.587, P236 = 0.534, P301 = 0.783, P321 = 0.873.

The number of positively-selected codons in the P ORF with non-synonymous substitutions was higher in the HBeAg-negative group than in the HBeAg-positive group, when either the two statistically significant codons were analysed ($p = 0.02$) or when the 11 codons with $\omega > 1$ were analysed ($p = 0.02$). There were no differences in the number of non-synonymous substitutions at any of the 15 codons with $\omega > 1$ between the groups with and without either the HLA-B*4001, HLA-B*5602 or HLA-A*02 family alleles (Supplementary Table 3).

We also used the www.immuneepitope.org algorithm to predict peptides in the P region that might be presented by these HLA class I alleles to CD8+ T cells. There were five potential HLA-B*4001-binding peptide-containing regions (aa 13–21, aa 135–143, aa 150–159, aa 538–548 and aa 703–710). The number of subjects with amino acid substitutions within these five regions was similar in the HLA-B*4001 (0, 0, 0, 0, 0) and HLA-B*5602 (0, 0, 2, 0, 0) subjects respectively. There were 25 regions containing potential HLA-B*5602-binding peptides and 44 regions containing potential HLA-A*02-binding peptides in the P ORF, including all six previously peptides identified by other authors (Rehermann et al., 1995). The number of subjects whose virus contained amino acid substitutions within these regions was also similar in subjects with and without these alleles (data not shown).

Table 6
Changes in intensity of positive selection in longitudinal data. Values are estimates of ω in the branches leading to the focal samples (foreground) relative to those in the remainder of the tree (background). Serum 1 predates Serum 2 by 5.2 ± 0.5 (mean ± sem) years.

HBV Gene	ω (PAML)		Number of positively selected codons (BEB)*	
	Serum 1	Serum 2	Serum 1	Serum 2
Core ($n = 7$[a])	2.4	4.7	0	2
S ($n = 8$)	9.3	5.0	2	0
pre-S ($n = 8$)	9.6	1.1	1	0
Polymerase ($n = 8$)	3.1	1.0	0	0

[a] A full-length core gene transcript could not be amplified from the baseline sample of one subject.
* $p > 0.95$ for $\omega > 1$ for codons 77 and 181 of the core gene and codons 204, 226 and 300 of the pre-S/S open reading frame.

Table 5
Codons in non-overlapping and overlapping (‡) regions of the polymerase gene which were under positive selection pressure ($\omega > 1.0$). A posterior probability of greater than 0.95 indicates statistical significance (i.e. codons 612 and 613).

Codon	Wild type amino acid	Posterior probability	Omega (±s.e.m.)	Subjects + ve at each site[a]	
				HBeAg +ve	HBeAg –ve
180	R/Q[b]	0.754	2.4 ± 0.9	1	3
612	I/V[b]	0.997	2.8 ± 0.5	1	9
613	L/R[b]	1.000	2.8 ± 0.5	0	12
665	Q	0.804	2.5 ± 0.8	0	4
671	S	0.569	2.0 ± 1.0	0	5
35[‡C169/170]	H	0.662	2.2 ± 0.9	0	3
283[‡pre-S102/103]	K/T[b]	0.701	2.3 ± 0.9	0	4
295[‡pre-S114/115]	Q/Y[b]	0.640	2.1 ± 0.9	1	2
302[‡pre-S121/122]	V	0.883	2.6 ± 0.7	1	3
828[‡X67/68]	G	0.571	2.0 ± 0.9	0	5
841[‡X80/81]	R	0.795	2.4 ± 0.8	0	3

[a] The number of subjects with at least one clone with a non-synonymous substitution.
[b] The wild type amino acid in sub-sub-genotypes C3A and C3B respectively.
[‡C] In the overlap with the C open reading frame.
[‡pre-S] In the overlap with the pre-S open reading frame.
[‡X] In the overlap with the X open reading frame.

3.6. Longitudinal analysis of selection pressure on core, envelope and polymerase genes

The within-subject changes in positive selection pressure over time are summarised in Table 6. Columns 2 and 3 show the ratio of non-synonymous to synonymous substitutions (ω) in each gene at each timepoint. This data indicates that an increase in ω was detected at the second timepoint in the core gene but not in the polymerase, pre-S and S genes. This increase in ω in the core gene was associated with an increase in the number of codons that came under statistically-significant positive selection pressure (columns 4 and 5). These were codons 77 and 181, both of which have been identified as coming under positive selection pressure in previously reported datasets (Abbott et al., 2010; Warner et al., 2011). We did not identify an increase in the number of codons coming under statistically-significant selection pressure in either the polymerase, pre-S or S genes.

4. Discussion

A signature of positive selection pressure on a gene is an elevated rate of non-synonymous substitutions relative to synonymous substitutions (ω); with the rate of synonymous substitutions taken as an estimate of the rate of random processes such as genetic drift. We have previously used the PAML software program to demonstrate an increase in positive selection pressure on a number of codons in the HBV core gene in the HBV from subjects in the IHC stage of a chronic HBV infection relative to subjects in the immune tolerant stage (Abbott et al., 2010; Warner et al., 2011). Statistical associations between substitutions at some of these codons and HLA class I alleles suggest that CD8+ T cells contribute to positive selection (Abbott et al., 2010). Together, these observations provide evidence that the immune mechanisms that put selection pressure on the HBV core gene have a significant role in suppressing HBV replication in most patients in the IHC stage of a chronic HBV infection; and thus will be useful targets of a therapeutic vaccine for chronic hepatitis B. However it is possible that suppression of replication may also require immune responses to epitopes outside the HBV core gene, as occurs in acute hepatitis B (Nayersina et al., 1993; Rehermann et al., 1996). The purpose of this study was to use phylogenetic analyses to assess the contribution of selection pressure on genes in the HBV pre-S/S and P open reading frames to viral suppression in IHCs.

We were unable to find evidence that selection pressure on pre-S/S ORF epitopes contributes to the suppression of HBV replication in most of the study subjects. Although there was evidence of selection pressure on 34 codons within the whole pre-S/S ORF, the trends for increased selection pressure in HBeAg-negative relative to HBeAg-positive subjects in cross-sectional data did not reach statistical significance, and there were no associations between non-synonymous substitutions in these codons and any HLA class I alleles. This contrasts with the previous study in the same group of subjects showing increased numbers of non-synonymous substitutions in positively-selected codons in the HBV core gene in HBeAg-negative subjects; some of which were associated with HLA class I alleles (Abbott et al., 2010). The small longitudinal study was consistent with these observations, in that increases with time in both omega (ω) and the number of codons under positive selection pressure were detected in the core gene but not in the pre-S/S open reading frame genes.

There are a number of possible explanations for the different pattern of selection pressure on the core gene and pre-S/S ORF genes. First, we tested subjects with a limited range of HLA class I alleles, and subjects with other alleles might have stronger anti-pre-S/S ORF responses. Second, the small Tongan population may have low frequencies of alleles at other polymorphic loci (e.g. KIR genes) that are necessary to mount NK cell or innate immune responses to pre-S/S ORF but not core antigens. Third, the mechanisms responsible for selection pressure on the pre-S/S ORF in acute HBV infection may be strongly suppressed in chronic HBV infection. For example, high viral load exhaustion due to S gene-rich subviral particles could suppress the anti-S gene activity of CD8+ T cells (Reignat et al., 2002) or NK cells (Tjwa et al., 2011) to a level that is difficult to reverse. Fourth, there might be substantial inter-individual variation in the ability to respond to pre-S/S ORF epitopes due to either unknown genetic or environmental factors. For example, subject N15 had non-synonymous substitutions in 10 of the 19 codons under selection pressure in the S gene, which contrasts with subjects N12, N13 and N17 who had no non-synonymous substitutions in the 19 codons. Thus a larger study might have found evidence of positive selection pressure against pre-S/S ORF epitopes in small sub-groups of subjects. Fifth, there might be substantial inter-individual variation in the epitopes within the pre-S/S ORF that are targeted by the immune system. The immuneepitope.org algorithm identified a large number of possible peptides within the pre-S/S ORF that could potentially be presented by HLA-A*02 family alleles. Our assay system relies on identifying positively-selected residues that are common to groups of subjects. If the CD8+ T cell responses to the pre-S/S ORF in each subject recognised a different peptide(s), we might not have detected them. However these results in Tongan patients are consistent with results obtained using immunological assays in European and American patients. Although CD8+ T cells responding to pre-S/S ORF peptides presented by HLA-A*02 alleles have been shown to exist in European and American subjects with a chronic HBV infection (Rehermann et al., 1996; Webster et al., 2004), their detection has not been associated with either control of viral replication or with the development of escape mutations (Webster et al., 2004).

These results raise the possibility that pre-S/S ORF antigens may have limited role in a therapeutic vaccine for chronic hepatitis B. Antigens that only induce immune responses in small subsets of individuals with CHB would have little value unless these subsets could be defined in advance using biological markers. It might be argued that powerful adjuvants could overcome any mechanisms that contributed to anergy or exhaustion of pre-S/S ORF-reactive immune cells, but these adjuvants would also carry the risk of inducing anti-self immune responses. It might be useful to look for evidence of anti-pre-S/S ORF CD8+ T cell epitopes in Asian IHCs with other common HLA class I molecules such as HLA-A*1101, -A*2402, -A*3303, -B*4601 and -B*5801 (Cao et al., 2001). Failure to find evidence of HLA class I-restricted selection pressure on the pre-S/S ORF in IHCs who were homozygous for these alleles would provide strong evidence that a therapeutic vaccine for CHB based on pre-S/S ORF antigens would not be successful in Asia. A cohort of 500 Han Chinese IHCs should be sufficient to find enough homozygous individuals to complete such a study. It is now possible to generate panels of HLA class I tetramers that contain a wide repertoire of peptide epitopes (Grotenbreg et al., 2008). Generation of a panel of HLA-A*02-based tetramers containing the full repertoire of 88 peptides from the pre-S/S ORF that are predicted to bind HLA-A*02 family alleles is possible. These could be used to test the hypothesis that some of the failure to find evidence of selection pressure on the pre-S/S ORF in IHCs is due to inter-individual diversity in the peptides recognised. Although published data from subjects with acute HBV infection suggests this is an unlikely possibility (Nayersina et al., 1993), it has not been excluded in IHCs. The best approach to the choice of antigens for a therapeutic vaccine for CHB may be to trial a vaccine containing the HBV core gene peptides that are presented by all the common HLA class I molecules that are commonly found in target populations. If such

a vaccine was successful, there would be no need to conduct further studies into pre-S/S and P ORF antigens.

There are a number of reasons to believe that selection pressure on the P ORF might contribute to suppression of viral replication in chronic HBV infection. Six polymerase peptides have been shown to activate CD8+ T cells in HLA-A*02-positive patients with acute hepatitis B (Rehermann et al., 1995; Webster et al., 2004); and CTL responses to some of these peptides have been associated with a successful response to interferon therapy for chronic hepatitis B (Rehermann et al., 1996). In addition, there is no reason to believe that exhaustion of these immune cells due high levels of antigen (as occurs with immune responses to the pre-S/S ORF Reignat et al., 2002) will occur. In contrast, the presence of these CD8+ T cells has not been associated with escape mutations in HBV-DNA, and it has been suggested that the levels of expression of P ORF peptides on the surface of hepatocytes may be too low for recognition by CD8+ T cells (Webster et al., 2004). Even though we found an overall increase in selection pressure on the P ORF in HBeAg-negative versus HBeAg-positive subjects, this was largely due to the high frequency of non-synonymous substitutions at codons 612 and 613 (see Supplementary Table 3). There was no increase in non-synonymous substitutions in the longitudinal data and no evidence of HLA class I restriction on any positively-selected codon. Thus our data are most consistent with the view that positive selection pressure on P ORF codons does not contribute strongly to suppression of viral replication in inactive healthy carriers.

It is not possible to identify the mechanisms responsible for the non-synonymous substitutions that were detected at positively-selected codons in this study. The repertoire of HLA class I alleles in our subjects included HLA-A*1101, HLA-A*2402 and HLA-A*3401, and statistical associations with these alleles could have been missed because of low statistical power. It is unlikely that promiscuous binding of peptides to both HLA-B*4001 and HLA-B*5602 was responsible for non-synonymous substitutions at the few sites that were commonly under positive selection pressure. These alleles differ by 28 amino acids in the exon 2 and exon 3 sequences that make up the peptide-binding groove, and immune-epitope.org analyses show markedly different peptide-binding repertoires. It is possible that immune mechanisms other than CD8+ T cells impose positive selection pressure on the pre-S/S and P ORFs. For example, antibody-driven escape mutations might account for the possible selection pressure on I126 which is in the a determinant of the S gene (Hsu et al., 2010). In addition, cytolytic CD4+ T cells (Brown, 2010), intracellular pattern recognition receptors, innate immunity and RNAi may all influence the outcome of infectious diseases. It is also possible that this study has overestimated the strength of selection pressure on pre-S/S and P ORF codons. There was no increase in the number of non-synonymous substitutions at positively-selected codons with time, raising the possibility that many of the non-synonymous substitutions at positively-selected sites may have been present in the HBV at the time of initial infection, having arisen in previously infected subjects.

It is of interest that there was no detectable selection pressure on a length of 302 P ORF codons that overlap the S gene, even though this region contains three HLA-A*02-restricted peptides that stimulate CD8+ T cells in acute HBV infection (Rehermann et al., 1995). This result could be unique to Tongan subjects with this limited repertoire of HLA class I molecules, or a random finding in a small study. Alternatively, protection of this large section of the polymerase gene from the immune system may be essential for the development of a chronic HBV infection. A therapeutic vaccine or drug that put selection pressure on any amino acids in these regions might be very successful in suppressing viral replication.

In summary, this data measures selection pressure on the pre-S/S and P open reading frames in subjects in the IHC stage of a chronic HBV infection. We could not identify selection pressure on any amino acid that was commonly associated with either the HLA-A*02, HLA-B*4001 or HLA-B*5602 alleles; which is consistent with previous studies of the HLA-A*02 allele (Martinet et al., 2012; Webster et al., 2004). Although these data sets are limited in size, they raise the possibility that peptide-based immunotherapies for CHB may not require peptides from the pre-S/S and P ORFs; and suggest the hypothesis that CD8+ T cells recognising peptides from the pre-S/S and P ORFs are not necessary for the development or maintenance of the IHC stage of a chronic HBV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.08.007.

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