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Are in vitro hepatitis B core promoter mutations important for clinical alterations in viral load?☆

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Abstract

In vitro studies of HBV core promoter mutations in hepatoma cell lines suggest that some mutations in core promoter transcription factor binding sites result in reduced core promoter activity and viral replication. We sought to validate this hypothesis using clinical samples with viral load differences before and after HBeAg seroconversion. A consensus sequence for transcription factor binding sites/regulatory regions was constructed based on published studies. Serum from two time points in 33 seroconverters and 10 interferon non-responders (controls) were utilized. Genotyping, HBV DNA quantification and direct sequencing of core promoter were performed. There were 216 new mutations following HBeAg seroconversion but few in controls. Mutations or mismatches to consensus transcription factor/regulatory region sequences clustered at nucleotide positions appeared genotype-specific, non-group specific or baseline mismatches and were discounted as having significant impact on viral replication. Only a few mutations in three seroconverters (9.1%) were specific, while 39.4% had no new mutations that could be attributed to reduction in viral load following HBeAg seroconversion. In 51.5% of patients, mutations were of uncertain significance because they occurred in demonstrated non-critical clustered nucleotide positions. Core promoter mutations post-seroconversion did not correlate with in vitro induced mutations that reduced the promoter activity.

Keywords: Hepatitis B virus; Core promoter; Mutation; Transcription factors; Hepatitis B e antigen seroconversion

1. Introduction

A high HBV viral load is implicated in disease pathogenicity and correlates closely with clinical hepatitis B disease such as hepatitis B flares or acute exacerbations (Liaw et al., 1988) and liver damage (Chan et al., 2002). Conversely, a reduction in viral load is associated with a remission in hepatic necroinflammatory activity and improved clinical outcomes following HBeAg seroconversion (Hsu et al., 2002) or therapy (Lai et al., 1998). A recent review demonstrated a strong correlation between hepatic activity index and HBV DNA levels (Mommeja-Marin et al., 2003), confirming the clinical impression that a high viral load was associated with high necroinflammatory scores, whilst a low viral load was associated with a reduction in hepatic activity index scores. The HBV viral load in serum represents a snapshot of the dynamics between rate of production of virions and the rate of removal of virions, presumably by the immune system (Perelson, 2002). While the immune system clearly plays a role in removal of HBV, particularly following HBeAg seroconversion, its role is complex and difficult to measure. Alternatively, the concept that mutations in the core promoter could affect viral replication is plausible as an 8-bp deletion was found to be associated with a reduction in viral replication (Kohno et al., 2000). In addition, in a study of mutant HBV variants, Bock and colleagues (Bock et al., 2000) demonstrated up to 10-fold reduction in viral replication using a luciferase assay system, with mutations in the binding site of hepatocyte nuclear factor (HNF) 3 and 4 thought to be responsible. Thus, it would seem that there

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is a basis for determining whether core promoter mutations are clinically relevant in altering viral replication.

Does core promoter activity have a central role in regulating viral replication clinically? While the production of HBV virions is complex, the core promoter is one of four key promoters regulating HBV viral replication (Kramvis and Kew, 1999). Numerous in vitro studies (Moolla et al., 2002) have shown that induced mutations within transcription factor binding sites in the core promoter region affect core promoter activity, viral replication and the production of viral particles. While the hypothesis that the HBV core promoter regulates viral replication seems reasonable, it has never been validated by clinical studies. The in vitro studies utilized transfection of experimental mutations or truncated constructs into hepatoma cell lines and core promoter activity was determined with or without measurement of viral replication. The basis for these studies has been the finding that numerous transcription factors in liver cells bind to different domains within the core promoter region—as has been reviewed by Moolla et al. (2002). These transcription factors comprise either negative or positive regulatory elements. The complexity of viral replication, however, can be illustrated by the A1762T/G1764A mutation, which leads to a reduction in precore mRNA. The same mutation concurrently generates a new binding site for HNF-1 that enhances the transcription of pregenomic RNA, leading to increased viral replication (Li et al., 1999). Core promoter mutations, particularly the A1762T/G1764A double mutation, have been associated with hepatitis B reactivation (Kajiya et al., 2002), fulminant hepatitis (Sato et al., 1995) and liver disease (Takahashi et al., 1999).

There have been few studies using clinically derived viral sequences of the HBV core promoter region. During HBeAg seroconversion, hepatic necroinflammatory activity resolves together with a 3–4 log reduction in viral load. There is loss of HBeAg and development of anti-HBe, and the process is associated with improved clinical outcomes (Hsu et al., 2002). Following HBeAg seroconversion, the residual viral strains represent those that have survived immune clearance, possibly with reduced replication competency, compared to the pre-seroconversion strains. Therefore, we sought to test the hypothesis that in vitro functionally demonstrated mutations in the core promoter region of the HBV genome are responsible for the reduced viral load following HBeAg seroconversion.

2. Materials and methods

2.1. Study design

A consensus sequence for the HBV core promoter transcription factor binding sites and regulatory regions was constructed after retrieving published studies from a PubMed search. Patients ($n = 33$) who had HBeAg seroconversion spontaneously or after interferon therapy were selected and interferon non-responders ($n = 10$) were used as controls. Genotyping of viral strains was performed by restriction fragment length polymorphism (RFLP) and HBV DNA quantification was performed using the Amplicor PCR assay. Direct sequencing was performed on pre- and post-seroconversion serum so that the dominant viral strains were analyzed. Individual viral sequences before and after seroconversion were then compared to each other to elucidate new mutations and compared to the consensus sequence to demonstrate mismatches.

2.2. Documentation of core promoter mutations

In order to determine as comprehensive a list of functionally important mutations as possible, a PubMed search was performed for all articles published using the MESH term "Hepatitis B core promoter" and it yielded 353 hits. Publications selected were then manually screened and selected to fulfill the following criteria: (i) non clinical strains of HBV were used, (ii) the core promoter region was used, (iii) core promoter activity was measured using a functional assay, (iv) site-directed mutagenesis or truncation experiments were performed to characterize specific mutations that affected core promoter activity, and (v) transfection experiments were performed in cell lines to ascertain core promoter activity. Articles fulfilling the above criteria were manually reviewed to determine mutations that affected core promoter activity. By integrating this data, a list of the types of important transcription factor binding sites/regulatory regions was obtained (Table 1), and a consensus sequence representing the accumulated critical nucleotides (wildtype) of these regions was constructed (Table 2). Alignment of sequences was then performed to ensure consistent numbering of the nucleotides.

2.3. Patients and sera

From hepatitis B patients on long term follow up at the National University Hospital, forty three Chinese patients with chronic hepatitis B who were initially HBeAg positive were selected and divided into three phenotypically distinct groups: IFN responders ($n = 16$) who achieved sustained HBeAg seroconversion after treatment with interferon; spontaneous seroconverters ($n = 17$) who seroconverted spontaneously; and IFN non-responders ($n = 10$) who did not seroconvert despite interferon treatment. One pair of sera samples from each seroconverter was studied, namely a pre- and post-seroconversion sample, while IFN non-responders had sera studied at a similar time interval after therapy. This study was approved by the Institutional Review Board of the National University Hospital, Singapore.

2.4. Hepatitis B investigations

Biochemical tests were carried out with routine automated methods. HBsAg, HBeAg, anti-HBe and anti-HBs were measured using microparticle enzyme immunoassay (MEIA, Abbott Laboratories, North Chicago, IL). All patients were negative for antibodies to hepatitis C virus (Quantiplex^{TM} 2.0 Assay, Chiron Corp, Emeryville, CA), hepatitis D virus and human immune deficiency virus (Abbott Laboratories, North Chicago, IL). Serum HBV DNA levels were quantified by an Amplicor HBV Monitor Test (Roche Molecular systems, Pleasanton, CA) following the Manufacturer's instructions. The dynamic range of this PCR based quantitative assay is from 4×10^2 to 4×10^7 copies per ml of serum.

Table 1
Domains of transcription factor (TF) binding sites/regulatory regions in the HBV core promoter, study systems and effects

Regulatory regions/TFs	DNA sequence of TF binding sites/regulatory regions in the core promoter[a]	Study system (fragments, vectors and cell lines used)	Effects
NRE	nt. 1603–1633, 5′-GTTGCATGGAGAC CACCGTGAACGCCCATCA-3′ (Lo and Ting, 1994)	Different fragments of WT/mutant HBV with the CAT reporter gene; different cell lines including Huh7, HepG2, HeLa and Vero cells	The deletion (nt. 1400–1682) increased the core promoter (CP) activity (Gerlach and Schloemer, 1992) but as this was a long fragment, further studies defined more specifically the NRE of the CP (Lo and Ting, 1994). A number of studies show that mutations involving the critical region of NRE that is within nt. 1613–1621 (corresponding to nt. 1611–1619) (Lo and Ting, 1994; Park et al., 1997; Sun et al., 2001) destroys most of NRE activity (and increases CP activity), but mutations in the NREγ sub-region nt. 1605–1621 (corresponding to nt. 1603–1619) (Chen and Ou, 1995) can also affect NRE activity
CURS/enhancer (EN) II	Core promoter upstream regulatory sequence (CURS) nt. 1634–1742, 5′-GATCCTGC-CCAAGGTCTTACATAA GAGGACTCTTGGACTC-CCAGCAATG TCAACGACCGACCTTGAG-GCATACT TCAAAGACTGTTTGTT-TAAGGACTG GGAGGAGTTG-3′ (Yuh et al., 1992)	Defined fragments of CURS/ENII and CP or other promoters with a CAT/luciferase reporter gene; different cells including HepG2, Hep3B, Huh6, Huh7, PLC/PRF/5, HA22T, and Hela cells	A 108 bp, nt. 1636–1744 (CURS, corresponding to nt. 1634–1742) (Yuh et al., 1992) or an 88 bp upstream fragment of the CP (Yee, 1989) activates the BCP in HepG2 and Huh7 or Huh6 cells. The CURS region overlaps with the ENII, which increased promoter activity >150-fold in Huh6 cells, about 15-fold in Huh7 and HepG2 cells (Su and Yee, 1992). The activity of the ENII is thought to require cooperation between A and B elements (nt. 1636–1690 and 1704–1741, corresponding to nt. 1634–1688 and 1702–1739) (Yuh and Ting, 1990). In a separate study, the part B (nt. 1687–1774, corresponding to nt. 1684–1771) of a 148 bp fragment (nt. 1627–1774 corresponding to nt. 1624–1771) was found to contribute 70% of entire ENII activity and comprised B1-B3 (nt. 1687–1705, 1706–1736 and 1737–1774) acting synergistically (Wu et al., 1992). Functional analysis indicates that a critical region appears to be within nt. 1640–1663 (corresponding to nt. 1638–1661) which corresponds to element A (Yuh and Ting, 1990), box α (Yuh and Ting, 1993) and “critical region for enhancer II activity”. This region appears to be also a binding region for transcription factors HLF, FTF, and E4BP4 (Ishida et al., 2000). Interestingly, the binding of E4BP4 to boxα results in repression of enhancer II activity (Lai and Ting, 1999). Studies (Wu et al., 1992; Yee, 1989; Yuh and Ting, 1990; Yuh et al., 1992) have demonstrated large fragments of HBV DNA do have enhancer activity, but the boundaries of the enhancer have not been well characterized, nor have binding sites of specific transcription factors been documented. Nonetheless these large fragments have been shown to be functionally important and thus we have included their large domains
RFX1	nt. 1603–1615, 5′-GTTGCATGGAGAC-3′ (Buckwold et al., 1997)	NRE (α, β and γ element), ENII and the CP with a CAT reporter; Huh7 cells	RFX1 can bind to NREγ and transactivates the CP (Buckwold et al., 1997)
Sp1	Site 1: 5′-TGAACGCCCA-3′ (nt. 1621–1630); site 2:5′-TGGGAGGAGT-3′ (nt. 1731–1740); site 3: 5′-GGGGAGGAGA-3′ (nt. 1743–1752) (Zhang et al., 1993)	One complete HBV genome with a luciferase reporter or a head-to-tail dimer of the HBV; Huh 7, HepG2, HepG2.1 and *D. melanogaster* Schneider line-2 cells	Sp1 binds to sites 1–3 and activates transcription in SL2 cells. Deletions (nt. 1737–1805 corresponding to nt. 1735–1803) result in loss of CP activity in HepG2 cells (Zhang et al., 1993). Mutations in regions consistent with Sp1-2 and Sp1-3 reduced the CP activity but this was more pronounced in undifferentiated hepatoma cells (Zhang and McLachlan, 1994). In a separate study, one nucleotide mutation in the three Sp1 binding sites abolished Sp1-1, Sp1-2 and Sp1-3 binding respectively and reduced the levels of the preC&pg RNA (Li and Ou, 2001)
FTF1/2	5′-CCAAGGTCT-3′ (nt. 1642–1650), FTF1; 5′-CGACCTTGA-3′ (nt. 1691–1699), FTF2 (Gilbert et al., 2000)	HBV ENI, WT/mutant CP and CAT gene; HepG2, Hep3B, PLC/PRF5 and HeLa cells	Mutations affected FTF site 2 or both 1and 2 reduced the CP activity in HepG2 cells co-transfected with expression vector FTF (Gilbert et al., 2000)

Table 1 (*Continued*)

Regulatory regions/TFs	DNA sequence of TF binding sites/regulatory regions in the core promoter[a]	Study system (fragments, vectors and cell lines used)	Effects
C/EBP	1: CCAAGGTCTTACATAA-GAGGACT (nt. 1642–1664); 2: CCCAGCAATGTCAACG (nt. 1673–1688); 3: GGAGGAGATTAGGTTAAA (nt. 1745–1762); 4: ACCAGCACCATGCAAC TTTTT (nt. 1805–1825): 5: CATATCATCTCT TG (nt. 1835–1848) (Lopez-Cabrera et al., 1990)	Defined fragments of HBV or ENII/CP with an SV40 promoter and a luciferase/CAT reporter; HepG2/2.2.1.5, CV1, Sk-Hep1, Huh7 and HeLa cells	A 12 bp (nt. 1645–1656 corresponding to nt. 1643–1654) and 71 bp (nt. 1591–1661 corresponding to nt. 1589–1659) deletions in C/EBP site 1 reduced the CP activity. The CP activity can be increased by low concentrations of C/EBP but repressed at higher concentrations using expression vectors in Hep G2 cells (Lopez-Cabrera et al., 1990, 1991). The C/EBP sites 1 and 2 have been shown to be functionally important in increasing enhancer II/pregenomic promoter activity (Choi et al., 1999). Binding assays using purified C/EBP could not demonstrate binding to C/EBP sites 3–5 contrary to previous publications (Lopez-Cabrera et al., 1990)
HNF1	nt. 1717–1735, 5′-GTTTGTTTAAGGACTGGGA-3′ (Wang et al., 1998)	Different scans of HBV ENII/CP with a CAT reporter; HepG2 and HeLa cells	HNF1 transactivated ENII and CP. ENII-B2 (nt. 1717–1735) was the target region of HNF1 (Wang et al., 1998)
HNF3	nt. 1713–1724, HNF3 site 5′-GACTGTTTGTTT-3′ (Johnson et al., 1995)	Two, three and four copies of different HBV oligos with the minimal TATA promoter and the luciferase gene or WT/mutant ENII/CP and CAT gene; Huh7, HepG2 and HeLa cells	The CP fragment (nt. 1713–1729 corresponding to nt. 1711–1727) can bind to HNF3β and the level of transcription increased >50-fold in the presence of exogenously expressed HNF3β (Johnson et al., 1995). Mutation G1717C reduced the activation of ENII and the CP by around 90% and mutations from nt. 1718–1722 reduced it by 80% (Li et al., 1995)
HNF4	Site 1: 5′-GGACTCTTGGAC-3′ (nt. 1660–1671); site 2: 5′-AGGTTAAAGGTCT-3′ (nt. 1755–1767) (Raney et al., 1997)	Serial deletions of HBV or WT/mutant CP with CAT gene; 1 copy of WT/mutant whole HBV DNA/CP with luciferase reporter; HeLa, Huh7, HepG2, HepG2.1, Hep3B, PLC/PRF5 cells	The HNF4 activated the CP about 20-fold in HeLa cells (Guo et al., 1993). Mutations of the site 1 (nt. 1660–1671) reduced the transcription while mutations of site 2 (nt. 1755–1767) increased the transcription from the CP in response to exogenous HNF4 in HepG2.1 cells. Mutations of site 1 and 2 eliminate HNF4-mediated transcription from the CP (Raney et al., 1997). The BCP activity of a mutant (with mutations within site 2) was reduced in Hep3B cells and HepG2 cells (Gilbert et al., 2000)
TBP	TA1: 5′-ATTA-3′ (nt. 1752–1755); TA2: 5′-TTAAA-3′ (nt. 1758–1762); TA3: 5′-TATTA-3′ (nt. 1771–1775); TA4: 5′-CATAAATT-3′ (nt. 1788–1795) (Chen et al., 1995)	Whole CP (adw, nt. 1634–1849) and the surface gene; Huh7 cells	Mutations in the TA1 to TA3 sites did not affect the levels of the pgRNA. Mutations in the TA4 greatly reduced the level of the pgRNA (Chen et al., 1995)
COUP-TF1/ HNF4α/PPARα-RXRα/TR	nt. 1755–1767, 5′-AGGTTAAAGGTCT-3′ (Yu and Mertz, 1997)	WT/mutant fragment/1.2 copies/a head-to-tail dimmer of HBV DNA with no reporter; Huh7, HepG2, H1299 and HepG2.1 cells	Some mutations in this TFs' binding site reduced the binding of COUP-TF1, HNF4, PPARα-RXRα, TR2, and TR4 (Li et al., 1999; Lin et al., 2003; Yu and Mertz, 1997, 2001) and abolished the function of these TFs on the transcription of the pgRNA and preC mRNA (Yu and Mertz, 1997) and reduced the transcription of both pgRNA and preC mRNA (Li et al., 1999; Yu and Mertz, 2001)

A total of 31 articles were surveyed based on a PubMed search and used to determine functionally validated consensus sequence (wildtype) for the transcription factor binding sites/regulatory regions in the HBV core promoter. A summary of mutations and their effects are also listed. TF: transcription factor; CP: core promoter; WT: wildtype; CAT reporter: chloramphenicol acetyl transferase reporter; pgRNA: pregenomic RNA; preC mRNA: precore mRNA; NRE: negative regulatory element; CURS/EN II: core upstream regulatory sequence/enhancer II region; C/EBP: CCAAT/enhancer binding protein; RFX1: regulatory factor for X-box 1; FTF: fetoprotein transcription factor; HNF: hepatocyte nuclear factor; Sp1: stimulating protein 1; COUP-TF: chicken ovalbumin upstream promoter-transcription factor; PPAR: peroxisome proliferators-activated receptor; RXR: retinoid × receptor; TR: human testicular receptor; TBP: TATA binding protein.

[a] The numbering of nucleotide positions has been adjusted after alignment.

2.5. Genotyping

Total DNA was extracted from each serum sample using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the Manufacturer's instructions, and genotyped (Lindh et al., 1998), using RFLP created by *Ava*II and *Dpn*II action on an amplified segment of the pre-S region. The pre-S region was amplified from the extracted DNA of serum sample by PCR using the primers: GP1 nt. 2823–2845; 5′-TCA CCA TAT TCT TGG GAA CAA GA-3′ and GP2 nt. 80–61; 5′-TTC CTG AAC TGG AGC CAC CA-3′.

2.6. Amplification of the HBV core promoter sequence

Nested PCR was carried out to amplify the core promoter region of HBV from the extracted DNA of serum samples, with all precautions taken to avoid contamination. The outer and inner primers were: P1, forward: 5′-CCG ATC CAT ACT GCG GAA-

Table 2

New mutations and mismatches to transcription factor (TF) binding sites/regulatory regions in the HBV core promoter

											CURS										
		NRE													C/EBP 1					C/EBP 2	
		RFX1			Sp1-1										FTF1						
Nucleotide		1605	1612	1613	1627	1628	1630	1631	1632	1633	1634	1635	1636	1638	1643	1652	1653	1655	1656	1673	1674
TF sites/regulatory regions		T	A	G	C	C	A	T	C	A	G	A	T	C	C	A	C	T	A	C	C
Patients	Genotype																				
N2	B	C-C						C-C		G-G			A-A			G-G					T-T
N3	B	C-C						C-C	G-C	G-G			A-A			G-G				T-T	T-T
N10	B	C-C					G-G	A-A				G-G		T-T							T-T
R1	B	C-C						C-C	G-G	G-G			A-A			G-G				T-T	T-T
R2	B	T-C		G-A				T-C		A-G			T-A	C-T		A-G				C-T	C-T
R3	B	C-C		A-G			A-C	C-C	C-G	G-G			A-A			G-G				T-T	T-T
R7	B	C-C						C-C		G-G			A-A			G-G				T-T	T-T
R8	B	C-C				T-C		C-C		G-G			A-A			G-G				T-T	T-T
R9	B	C-C		A-A				C-C		G-G			A-A			G-G			G-A	T-T	T-T
R10	B	C-T						C-C		G-G			A-A			G-G				T-T	T-T
R12	B	C-T						C-C		G-G			A-A			G-G				T-T	T-T
R13	B	C-C						C-C		G-G			A-A			G-G				T-T	T-T
R14	B	C-C						C-C	G-C	G-G			A-A			G-G				T-T	T-T
R15	B	T-C						T-C		A-G			T-A			A-G				C-T	C-T
S1	B	C-T		A-G				C-C		G-G			A-A			G-G			C-A	T-T	T-T
S2	B	C-C		A-G				C-C		G-G			A-A			G-G				T-T	T-T
S4	B	C-C		G-A						G-G			A-A			G-G		C-C		T-T	T-T
S5	B	C-C						C-T	G-G	G-G			A-A			G-G				T-T	T-T
S7	B	C-T						C-C		G-G			C-A			G-G				T-T	T-T
S8	B							C-C													
S9	B	C-C						C-C		G-G			A-A			G-G				T-T	T-T
S10	B	C-C					A-C	C-C	A-G	G-G			A-A			G-G				T-T	T-T
S11	B	C-T						C-T		G-A			A-T			G-A				T-C	T-C
S13	B	T-C						T-C	C-G	A-G			T-A			A-G				C-T	C-T
S14	B	C-T						C-C		G-G			A-A			G-G				T-T	T-T
S15	B	C-C		A-G				C-C	T-T	G-G			A-A			G-G				T-T	T-T
S16	B	C-T		G-A				C-C	G-G	G-G			A-A			G-G				T-T	T-T
S17	B	C-C		A-G				C-C	C-T	G-G			A-A			G-G				G-G	T-T
N1	C	C-C			T-T			C-C				G-G		T-T							
N4	C	C-C						C-C				G-G		T-T						T-T	
N5	C	C-C						C-C				G-G		T-T							
N6	C	C-C	C-C					C-C				G-G		T-T							
N7	C	C-C		A-A				C-C				G-G		T-T							
N8	C	C-C						C-C				G-G		T-T							
N9	C	C-C					G-G	A-A				G-G		T-T							
R4	C	C-C		G-A			G-G	A-A			G-A	G-G	C-T				C-T				
R5	C	C-C					G-C	C-C	C-G	A-G		G-A	T-A	T-C	T-C	A-G				C-T	
R6	C	C-C						C-C		A-G		G-A	T-A	T-C		A-G				C-T	
R11	C	C-T					G-A	A-C		A-G		G-A	T-A	T-C		A-G				C-T	
R16	C							T-C		A-G			T-A			A-G				C-T	C-T
S3	C	C-C		G-A			G-G	C-C				G-G		T-T							
S6	C	C-C		G-A			A-G	C-A				G-G		T-T						T-C	
S12	C	T-C					A-G	T-C				A-G		C-T							C-T

		CURS																HNF4-2, RXR, PPAR COUP-TF1, TR						
		C/EBP 2						HNF3-2																
			HNF3-1					HNF1						Sp1-2		Sp1-3							TA3	
Nucleotide		1676	1678	1688	1701	1702	1703	1719	1721	1725	1726	1727	1730	1739	1740	1745	1752	1757	1758	1760	1762	1764	1773	1775
TF sites/regulatory regions		A	C	G	G	C	A	T	G	A	A	G	C	G	T	G	A	G	T	A	A	G	T	A
Patients	Genotype																							
N2	B							G-G				T-T	G-G										C-C	
N3	B							G-G			A-C	T-T	G-G			T-G	G-A						C-C	
N10	B								A-A			A-A						A-A					C-C	G-G
R1	B							G-G			A-C	T-T	G-G				G-G						C-C	
R2	B						C-A	G-G			A-C	G-T	C-G		C-T								T-C	
R3	B							G-G			C-A	T-T	G-G										T-C	
R7	B							G-G			C-C	T-T	G-G										C-C	
R8	B							G-G			C-A	T-T	G-G				T-A						C-C	
R9	B			A-G	C-G	A-C		G-G			C-C	T-T	G-G				A-G						C-C	
R10	B	G-A						G-G				T-T	G-G										C-C	
R12	B							G-G			C-A	T-T	G-G											
R13	B							G-G			A-C	T-T	G-G										C-C	
R14	B							G-G			C-A	T-T	G-G				G-A						C-C	
R15	B						C-A	G-G				G-T	C-G		C-T								T-C	
S1	B							G-G				T-T	G-G										C-C	
S2	B							G-G			C-C	T-T	G-G										C-C	
S4	B							G-G				T-T	G-G										C-C	
S5	B							G-G			C-A	T-T	G-G										C-C	
S7	B							G-G			C-A	T-T	G-G				G-A				T-A	A-G	C-C	
S8	B						C-C	G-G							C-C								C-C	
S9	B							G-G			C-A	T-T	G-G								T-A	A-G	C-C	
S10	B							G-G			C-A	T-T	G-G				G-A				T-A	A-G	C-C	
S11	B						A-C	G-G		G-A	C-A	T-G	G-C		T-C								C-T	
S13	B						C-A	G-G				G-T	C-G		C-T		A-G						T-C	
S14	B							G-G			C-A	T-T	G-G										C-C	
S15	B							G-G			C-C	T-T	G-G										C-C	
S16	B		Ins					G-G			C-C	T-T	G-G				Del	Del	Del	Del	Del	Del	C-C	
S17	B							G-G			C-C	T-T	G-G										C-C	
N1	C																						C-C	
N4	C													A-A			T-T		C-C				C-C	
N5	C																				T-T		C-C	
N6	C																						C-C	
N7	C	T-T										A-A								G-G			C-C	
N8	C											A-A											C-C	
N9	C								A-A			A-A						A-A					C-C	G-G
R4	C							G-G	A-A			A-A									T-T	A-A	C-C	G-A
R5	C							G-G	A-G			A-T	C-G								T-T	A-A	C-C	G-A
R6	C							G-G			A-C	A-T	C-G						C-T				C-C	
R11	C							G-G	A-G		C-A	A-T	C-G					A-G					C-C	G-A
R16	C						C-A	G-G				G-T	C-G		C-T								T-C	
S3	C							G-G	A-A												T-T	A-A	C-C	G-G
S6	C							G-G	G-A			A-A						G-A			A-T	G-A	C-C	A-G
S12	C						C-A	G-G				G-A			C-T			G-A			A-T	G-A	T-C	A-G

The consensus sequence of the TF sites/regulatory regions is displayed under the nucleotide positions. The baseline nucleotide is shown as the first letter and the nucleotide in the post-seroconversion sample is the second letter at each position. Patient coding: N, interferon non-responder; R, interferon-induced seroconverter; S, spontaneous seroconverter. Only mutations/mismatches to the TF consensus are shown. Type A mutations: (green) Specific new mutations to the consensus sequence at the second time point; (red) new mutations at second time point of uncertain significance. Type B mutations or mismatches comprising: (blue) mutations occurring only at baseline that mismatch the consensus sequence, (purple) new mutations resulting in mismatches to the consensus in the baseline and second time point samples, (□) identical nucleotides at both time points which mismatch the TF consensus sequence. (yellow) Sites with genotype related nucleotide changes; (orange) sites with non-group specific mutations/mismatches. NRE: negative regulatory element; CURS/EN II: core upstream regulatory sequence/Enhancer II region; C/EBP 1, 2: CCAAT/enhancer binding protein, binding sites 1 and 2; RFX1: regulatory factor for X-box 1; FTF: fetoprotein transcription factor; HNF3-1,2: hepatocyte nuclear factor 3 binding sites 1 and 2; HNF4-2: hepatocyte nuclear factor 4, binding site 2; Sp1-1,2,3: stimulating protein 1, binding sites 1,2,3; COUP-TF: chicken ovalbumin upstream promoter-transcription factor; PPAR: peroxisome proliferators-activated receptor; RXR: retinoid × receptor; TR: human testicular receptor; TA3: third TA rich region.

3′ and P2, reverse 5′-GAG AGT AAC TCC ACA G-3′ for the first round PCR; P3, forward 5′-CGT GTG CAC TTC GCT TCA CCT-3′ and P4, reverse 5′-CAA AGC CAC CCA AGG CAC-3′ for the second round PCR. Amplification was carried out with primers P1 and P2 and 5 μl of DNA using *Taq* DNA polymerase (Hot Star, Qiagen, Hilden, Germany) with an initial activation at 95 °C for 15 min followed by 35 cycles of DNA denaturation at 94 °C for 1 min, annealing of primers at 50 °C for 1 min, and extension at 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 7 min. For the second round of PCR, 5 μl of the first round of PCR product was added with primers P3 and P4 with the same amplification conditions except for the annealing of primers at 55 °C.

2.7. Sequencing of the HBV core promoter

PCR products were purified using the GENECLEAN Kit (Qbiogene, Carlsbad, CA) according to the Manufacturer's protocol. Sequencing of the purified PCR products were carried out using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) following the Manufacturer's instruction. The inner primers P3 and P4 were used for the sequencing reactions on the ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA). The core promoter sequences (nt. 1591–1822) were aligned using the program DNA Star (DNASTAR Inc., Madison, WI) and analyzed. Numbering of the HBV sequence begins at the nucleotide after the two A in the unique but non-conserved *Eco*RI site of the HBV reference sequence (Accession No.: NC 003977) in the NCBI Gene bank.

2.8. Statistical analysis

The pre-seroconversion and the post-seroconversion sequences were compared to each other and to the reference core promoter consensus sequence of transcription factors binding sites/regulatory regions (Table 2) and categorized as new mutations or mismatches or combinations thereof. HBV DNA results were presented as mean ± S.E.M., and analyzed by one-way analysis of variance (ANOVA) between groups and paired *t*-test for related samples between time points. Categorical variables (genotype and frequency of mutations) were tested by χ^2 analysis and Fisher's exact test. In all tests, the p values of less than 0.05 were considered statistically significant. All analyses were carried out using SPSS for Windows (version 11.0).

3. Results

3.1. Characterization of published functionally important core promoter mutations

A total of 31 articles were found to fulfill the requirements generated in the search. The list of transcription factor binding sites/regulatory regions and mutations that were found to be functionally significant is shown in Table 1.

3.2. Clinical characteristics of patients in this study

There was no difference in the sex ratio, mean age and mean ALT levels between different groups of patients. Spontaneous seroconverters, IFN responders and IFN non-responders had a mean age of 36 ± 1.4, 32.7 ± 2.8, and 30.1 ± 2.1 years, respectively, baseline mean ALT of 140.7 ± 41.8, 235.7 ± 99, and 62.1 ± 11.5 IU/ml, respectively, and baseline HBV DNA of 7.6 ± 0.26 ($\log_{10}$ copies/ml), 7.9 ± 0.26 ($\log_{10}$ copies/ml) and 8.5 ± 0.33 ($\log_{10}$ copies/ml) respectively. Of the 43 chronic hepatitis B patients, 28 (65.1%) were genotype B and 15 (34.9%) were genotype C. The prevalence of genotype B and C in IFN responders (68.8% and 31.2%) was similar to spontaneous seroconverters (82.4% and 17.6%). However, there was a higher prevalence of genotype B in seroconverters (75.8%) than in non-seroconverters (30%) ($p < 0.05$). Seroconverters with normalized ALT had a significant reduction in HBV DNA level by a mean of 3.8-log after HBeAg seroconversion compared to the pre-seroconversion samples, while ALT and HBV DNA levels remained unchanged in IFN non-responders. HBV DNA levels between spontaneous seroconverters and IFN responders were similar.

3.3. Classification of mutations and mismatches

If we hypothesize that core promoter transcription factor binding sites are important regulators of viral replication, then following HBeAg seroconversion, we would expect new mutations to occur in such sites (Type A). All other mutations or mismatches to the consensus binding site sequence would be of little relevance (Type B, Table 3).

3.4. Overall new mutations and mismatches

All seroconverters (33/33) but only one non-seroconverter (1/10) had new mutations ($p < 0.001$) compared to their baseline samples. Overall, 220 new mutations occurred in 46 of 232 nucleotides of the core promoter in 43 chronic hepatitis B patients and most of them (188/220, 85.5%) were located within transcription factor-binding sites/regulatory regions. There were only four nucleotide positions where mutation occurred in more than 30% of patients (nt. 1605, 1613, 1636 and 1726) (Fig. 1). Spontaneous seroconverters and IFN responders had similar mutation rates (119/220, 54.1% and 97/220, 44.1% respectively), whilst IFN non-responders had few new mutations (4/220, 1.8%) ($p < 0.001$). Compared to the consensus sequence of the transcription factor binding sites/regulatory regions, many mismatches were present in both baseline and post-seroconversion samples. These mutations and mismatches were clustered at some positions and appeared to be genotype-specific or non-group specific (Tables 2 and 3).

3.5. Type A mutations

The most convincing mutations likely to affect core promoter activity are new mutations that occurred post-seroconversion (second time point) in the transcription factor binding sites

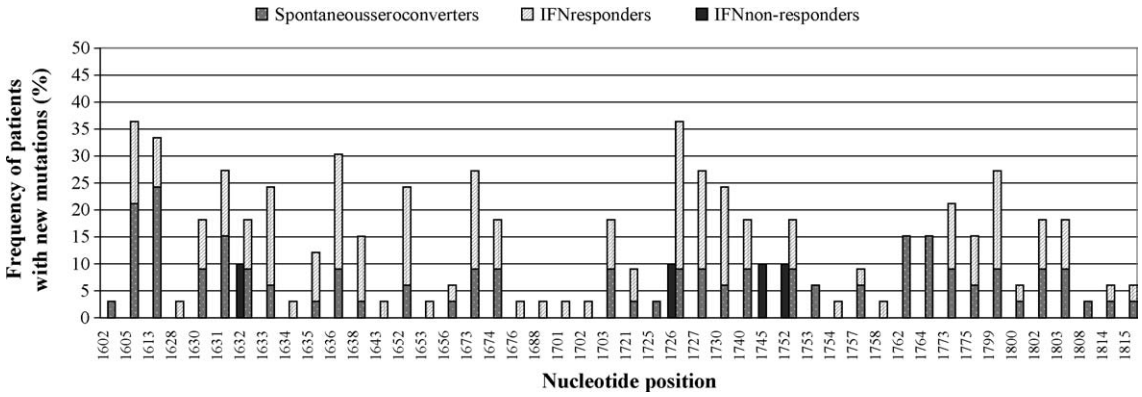

Fig. 1. Frequency of patients with new mutations in the HBV core promoter sequence. New mutations were observed at 46 sites of the HBV core promoter when the sequences of baseline and post-seroconversion (second time point) samples were compared. Most of these mutations occurred in seroconverters (spontaneous or IFN induced) rather than IFN non-responders. There were only four sites at which new mutations were present in over 30% of seroconverters.

(red and green highlights in Table 2). Although 20/33 (60.6%) of seroconverters compared to IFN non-responders (1/10) ($p < 0.01$) showed mutations of this nature, many of these shared characteristics of genotype-specific and non-group specific mutations or were present in the baseline sample of other seroconverters. They were observed in 17/33 (51.5%) of seroconverters and are not likely to affect core promoter activity. Consequently, there were only four specific mutations at three sites observed following HBeAg seroconversion in 3 (9.1%) of seroconverters (green highlights in Table 2), including one patient (R4) having G1634A (in the CURS) and C1653T (in the C/EBP 1 domain) and two patients (R3 and S10) with the same mutation A1630C (in the Sp1-domain 1), that could be confidently attributed to altered core promoter activity.

Table 3
Categorization of Type B mutations/mismatches and clustering sites

Type B mutations/mismatches	Location (nucleotide, nt.) in Table 2
Mismatches occurring only in baseline sample compared to the consensus sequence	Blue boxes (▭)
New mutations resulting in nucleotide mismatches to the consensus sequence at both time points	Purple boxes (▭)
Identical nucleotides at both time points which mismatch the consensus sequence	Uncolored boxes with letters (□)
Clustering sites	
Genotype-specific (sites bearing mutations correlating closely with genotypes)	Sites in light yellow box: nt.1633, 1635, 1636, 1638, 1652, 1673, 1674, 1727 and 1730 (▭)
Non-group specific (sites with mutations occurring across all clinical groups including non-seroconverters)	Sites in the orange boxes: nt.1605, 1631 and 1773 (▭)

Classification of type B mutations, which are considered non-significant in the context of this study. These mutations either are already present at baseline and mismatch the consensus sequence of the HBV core promoter (the presence of a high viral load at baseline makes these unlikely to be contributory), or they occur at specific clustered sites which appear to have no relationship to changes in viral load following seroconversion, but are either related to genotype or are not group specific.

3.6. Type B mutations/mismatches

A variety of other mutations or mismatches were found in the transcription factor binding sites in the HBV core promoter region. These are classified in Table 3, and are either of uncertain significance, or not of importance in contributing to core promoter activity. Mismatches occurring in only baseline samples compared to the consensus sequence of the transcription factor binding sites (blue in Table 2) suggest that such mutations were not important in decreasing viral replication, since viral load was high at baseline. Patients with new mutations occurring following seroconversion that mismatch the consensus sequence at both baseline and post-seroconversion (purple highlights in Table 2) were of uncertain significance. Nucleotides that were identical at both baseline and post-seroconversion but which nevertheless were mismatches to the consensus sequence (Tables 2 and 3) would not contribute to viral load change following seroconversion since the nucleotide did not change over different time points.

3.7. A1762T/G1764A double mutation

This double mutation was only observed in five patients, only two of which were new mutations following HBeAg seroconversion (Table 2), while the other three were present at baseline in the patients.

4. Discussion

The postulate that in vitro induced core promoter mutations in the transcription factor binding sites/regulatory regions are important in regulating viral replication requires clinical validation. Our study is the first to examine this subject, and has shown that 39.4% of the patients had no new mutations in the transcription factor binding sites/regulatory regions of the core promoter following HBeAg seroconversion, and therefore this cannot be the reason for the reduction in viral replication. For

the remaining 60.6% of patients, although new mutations were found in the transcription factor binding sites/regulatory regions of the HBV core promoter, only at three sites in 9.1% patients (R3, R4 and S10) were the mutations specific and likely to have affected viral transcription through core promoter activity. These mutations affected the CURS (Yuh et al., 1992), the binding site C/EBP-1 (Lopez-Cabrera et al., 1990) and the binding site Sp1-1 (Zhang and McLachlan, 1994), all shown to affect core promoter activity. In the remaining 51.5% cases, the significance of mutations following HBeAg seroconversion is questionable since these mutations did not create a mismatch in the transcription factor binding sites associated with reduction in viral load. Functional studies would be needed to single out the effects of multiple mutations found in our study on core promoter activity and viral replication. The findings of our study showed that although mutations in the HBV core promoter region are common following HBeAg seroconversion, only in three (9.1%) patients can they be clearly associated with a decrease in viral replication.

Consequently, mutations that reduce core promoter activity or alter pregenomic RNA in vitro should be cautiously interpreted as it is unclear whether they are clinically meaningful. In order for clinically meaningful data to be obtained, samples from well characterized clinical phenotypes need to be used for in vitro functional experiments. One such study (Parekh et al., 2003) used high and low viremia samples in a cross sectional study of genotype A HBeAg positive patients. They found an association between core promoter mutants and low viremia in serum of their patients, but discordance between experimental in vitro results from cell culture and in vivo viral load. Patients with low viremia developed high replicator strains, and, conversely, patients with high viremia developed low replicator strains when transfected into hepatoma cell lines. The explanation for this discrepancy between cell models and in vivo clinical samples is unclear, but could be due to differences in transcription factor expression in hepatoma cell lines or higher removal of viral strains in patients with increased immune response, such as those following HBeAg seroconversion. In vitro methods generally utilize viral replication in hepatoma cell lines as a surrogate to reflect viral load in patients. However, until a validated cell model is found that correlates closely with clinical viral load, evaluation of replication efficiency in cell models cannot be inferred with confidence as reflecting altered in vivo viral load. Alternatively, cellular production of virus may be unchanged or even enhanced, but the immune removal of virus could be more efficient after seroconversion, thus resulting in a net viral load that is low.

Core promoter transcription factor binding sites are embedded within regulatory elements (Kramvis and Kew, 1999) (Table 2), making interpretation of their functional significance difficult. There appears to be a high degree of complexity in the regulatory process or viral replication, which may be lost by investigating each transcription factor or regulatory region in isolation. The use of phenotypically undefined viral strains, differences in experimental design, deletions, lack of measurement of pregenomic RNA, differences in cell lines and methods of measurement of either viral replication or promoter activity may explain the lack of correlation between our clinically derived mutations and those induced experimentally.

Clinically derived core promoter mutations such as the A1762T/G1764A mutation (Kramvis and Kew, 1999) and deletions also show conflicting effects on viral replication (Chen and Oon, 2000; Kohno et al., 2000). Taken together, this raises the question of whether core promoter activity really reflects in vivo viral load. Due to the differentially regulated core promoter transcripts (Yu and Mertz, 1996), only studies measuring pregenomic RNA or productive virus can address this issue. Clinically derived core promoters do appear to have a good correlation with core promoter activity and virus produced from HepG2 cells, but a poor correlation with in vivo viral load (Chun et al., 2000), a finding consistent with our results, suggesting that the core promoter has a questionable role in clinical regulation of viral replication.

Other than the core promoter, mechanisms that alter viral replication include increased encapsidation (Baumert et al., 1998), the postulated "Kozak sequence" around ATG start codons that increase translation (Kozak, 1986), and defective virion secretion due to mutations in the core region (Le Pogam et al., 2000). Alternatively, active and continuing immunological clearance may result in a low viral load despite a high production of viral particles. Sophisticated immunological studies would be needed to verify this issue. From our study it appears that HBV core promoter mutations that affect transcription factor binding sites/regulatory regions do not appear to have a role in reduction of in vivo viral load in a significant proportion of patients. Our findings confirm the complexity of regulation of viral replication of HBV and the discordance between in vitro cells based models and clinical in vivo viral load, and suggests that genomic elements outside the core promoter region may play an important role. Whichever domain explored would require validation using an in vitro cell based model of HBV replication that correlates closely with in vivo viral load, for such results to be clinically meaningful. Consequently, investigators using in vitro models to determine the effect of core promoter mutations should take caution and use phenotypically characterized, clinically derived samples.

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