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Methylation Regulates Hepatitis B Viral Protein Expression

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Abstract

Background—Hepatitis B (HBV) DNA has been shown to contain CpG islands that are methylated in human tissues, suggesting a role for methylation in regulating viral protein production. However, data are lacking on whether methylation regulates viral gene expression.

Methods—To investigate the hypothesis that methylation of viral DNA regulates viral gene expression, unmethylated, partially methylated, and fully methylated viral DNA were transfected into HepG2 cells. In addition, a new assay was designed that specifically identifies methylated cccDNA in human liver tissues.

Results—Transfection of methylated HBV DNA led to reduced HBV mRNA levels, decreased surface and core protein expression in cells, and decreased secretion of HBV viral proteins into the cell supernatant. These data provide direct evidence that CpG islands regulate gene transcription of HBV. Furthermore, methylated cccDNA was found in benign and malignant human liver tissues. Finally, an in-vitro equivalent of cccDNA showed decreased viral protein production in HepG2 cells after DNA methylation.

Conclusions—Together, these data demonstrate that methylation of viral CpG islands can regulate viral protein production.

Keywords

Hepatitis B; methylation; CpG; cccDNA

Background

More than 350 million persons are estimated to have chronic hepatitis B virus (HBV) infection, which can be identified by the persistent detection of the HBV surface antigen (HBsAg) in the blood [1]. Expression of viral proteins within host tissues are necessary for viral replication and the development of persistent infection. HBV protein expression is regulated by both cis-acting elements encoded within the HBV genome and by various trans-activating host factors. The cis-acting mechanisms include promoters for the precore/pregenomic transcripts, the preS1 transcript, the preS2/S transcript, and the X transcript. HBV DNA also contains two enhancers [2], as well as a negative regulatory region [3]. These regulatory regions can operate both independently as well as in co-operation with

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each other to regulate viral protein production and viral replication. For example, tissue specificity of HBV replication is in part determined by the combined effects of the core promoter as well as enhancer I [4]. All of these cis-acting elements depend on host transcription factors for their function [5,6]. Thus, they are likely to interact with host transcription factors via similar mechanisms to which human DNA interacts with transcription factors. This observation raises the possibility that additional host regulatory mechanisms that are active in the human genome may also be relevant to regulation of HBV gene expression.

In human tissues, gene expression can be regulated by methylation of CpG islands, which typically down regulates mRNA and protein production. Whether methylation of CpG islands regulates HBV genes is unknown. HBV DNA can be methylated in human tissues in both non-integrated forms [7] and following integration into the human genome [8]. However, data demonstrating that HBV CpG islands are functional and regulate viral protein expression when methylated are limited. In this study we demonstrate that methylation of viral DNA regulates viral gene expression and that methylated HBV cccDNA is present in human tissues.

Methods

Cell culture system

We used the system reported by Gunther et. al. [9] as a model of HBV infection. As an overview, a cloned HBV genome is released from the vector and transfected into HepG2 cells as a 3.2 kb linear DNA fragment containing the complete HBV genome. Once inside the HepG2 cells, the linear DNA can be circularized by host enzymes forming cccDNA. We modified this system for some experiments by in-vitro methylation of the linear HBV DNA prior to transfection as well as by in-vitro formation of the equivalent of cccDNA prior to transfection.

The full length genome of HBV was cloned (Stratagene) from the serum of an individual with chronic HBV infection. The serum was HBeAg and HBsAg positive and serum DNA levels were 7.4 log/ml. Sequence analysis demonstrated the virus was genotype D. Nucleotides are numbered from the EcoRI digestion site.

Methylation of HBV DNA

The cloned virus was digested with *Sap I* to release the HBV DNA from the vector and the vector was removed by gel electrophoresis. Following this, the HBV DNA underwent in-vitro methylation with *CpG Methyltransferase* (*M.SssI*, New England Biolabs). This enzyme methylates the cytosine in all CpG dinucleotides. Successful methylation was confirmed using methylation sensitive restriction enzymes that cut only unmethylated DNA (*HhaI* and *HpaII*, New England Biolabs). The control unmethylated DNA completely digested while no digestion was seen with the methylated DNA. The integrity of the methylated DNA was confirmed by gel electrophoresis and by full length PCR amplification of the methylated DNA. In addition, the methylation status of islands 1 and 2 following transfection were investigated by bisulfite sequencing and cloning of the HBV DNA at 48 hours following transfection. No loss of methylation was observed (data not shown).

The density of methylation in HBV CpG islands can range from low to heavy levels of methylation [7]. To investigate whether low levels of methylation could also affect gene expression, additional experiments were performed after in-vitro methylation with *HpaII Methyltransferase* (New England Biolabs) which methylates CpG dinucleotides in the context of 5’-CCGG-3’.

Transfection of HepG2 cell lines

HepG2 cells (American Type Culture Collection) were seeded at a density of 5 log cells in standard 24 well plates and grown overnight in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum and transfected (Lipofectamine 2000, Invitrogen) with 10 to 12 log copies of methylated or unmethylated HBV DNA. Twenty-four hours after transfection, the cells were washed and the growth medium replaced. Supernatant and cells were then separately harvested at 48 hours. Each transfection experiment comparing unmethylated to methylated HBV DNA was performed in duplicate wells with at least three independent experimental replicates.

The amount of input HBV DNA was measured by real time PCR prior to transfection. In control experiments, transfection with different copy numbers of unmethylated HBV DNA showed a linear relationship between the amount of DNA input and HBsAg and HBeAg production in the supernatant over the input range of approximately 9 to 12 log HBV genome equivalents/well (R^2=0.91 for HBsAg and 0.74 for HBeAg). Levels greater than 12 log copies of HBV appeared to saturate the system while levels less than 9 log gave low and variable results. Thus, all experiments were performed using HBV DNA input within the linear range and findings normalized for the amount of DNA input. By ANOVA, there was no statistical difference in the average amount of transfected DNA between the groups, p= 0.1, with average inputs of 10.17, 10.51, and 10.64 log HBV DNA/well for the unmethylated, fully methylated, and partially methylated groups respectively.

HBV mRNA detection

For mRNA studies, three separate wells were studied from each experimental condition. Total RNA was extracted from cells using TRIzol (Invitrogen) followed by precipitation with isopropyl alcohol as per manufacture's protocol. RNA extracts were DNAse I treated (Invitrogen) and cDNA was synthesized with oligo-dT primers using the Superscript First – Strand synthesis system for RT-PCR (Invitrogen). The model system in this study uses large amounts of HBV DNA and DNAse treatment does not remove all DNA in all cases, as demonstrated by signal positivity in samples processed without RT enzyme. However, in experiments with unmethylated HBV DNA, the mRNA signal was at least 100 times greater than the residual DNA signal, indicating that residual DNA contributed less then 1% to the quantification of the HBV mRNA.

Real time PCR was performed with the SmartCycler system (Cepheid) using the Fast Start SYBR green master mix (Roche). One μl of cDNA was used as input template for each real time PCR reaction. Primers are shown in Table 1. PCR cycling conditions were 10 minutes at 95°C followed by 40 cycles of 95°C for 20s, 55°C for 25s, and 72°C for 25s. Specificity was confirmed by melt curve analysis and band size in 1% agarose gels. Primers directed against the core and surface HBV gene transcripts were used. Because of the nature of the HBV genome, both assays measure the combination of their target plus other overlapping transcripts such as the pre-genomic transcript (Table 1).

Levels of mRNA were quantitated using an absolute standard curve. Data was normalized to the amount of cccHBV DNA to control for the amount of successful circularization. Data were further normalized for the total amount of cellular DNA to control for any subtle differences in the number of cells.

HBV protein detection

HBsAg and HBeAg ELISA assays (ETI-MAK-2 plus and ETI-EBK plus, Diasorin) were preformed on 100 ul of supernatant as per the manufacturers instructions. In one set of experiments, cells were fixed in formalin, paraffin-embedded and routine

immunohistochemistry performed for HBsAg and HBV core antigen (HBcAg). Antibodies (Dako) were used at a 1:25 and 1:900 dilutions respectively.

Student t-tests were used to test differences in mRNA and protein expression amongst groups.

Studies of HBV cccDNA

We also sought to determine if cccDNA was methylated in human liver tissue. Primers for bisulfite sequencing were designed to target a portion of island 2 and to be specific only for cccDNA. To accomplish this, we focused on the portion of the HBV genome where the positive strand is incomplete in non-cccDNA, with primers spanning from nucleotide positions 1377 to 1674 (Table 1). We exploited the fact that after bisulfite conversion, positive and negative strands of DNA are no longer complementary and designed bisulfite sequencing primers that amplify only the positive strand of DNA. Since the positive strand is present in this region in cccDNA, methylation of cccDNA can be detected. The success of this amplification strategy was confirmed by cloning and sequencing the PCR product which showed it to be positive strand in all cases. Twelve liver tissues from eight individuals with chronic HBV infection were then studied using the cccDNA bisulfite sequencing primers. Use of human tissue was pre-approved by the Institutional Review Board. All cases were negative for HBV DNA integration by the Alu-HBV PCR assay [10]. DNA was extracted from 20-25 mg of liver tissue (QIAmp DNA mini kit, Qiagen) and bisulfite treated (EpiTect Bisulfite Kit, Qiagen), amplified with cccDNA specific primers (Table 1), and cloned. Five to eight clones were submitted for sequencing.

In order to determine the effect of methylation on HBV cccDNA, linear cloned HBV DNA was ligated in-vitro to form closed circular loops that would be equivalent to cccDNA. This DNA was then methylated with *CpG methyltransferase* (New England Biolabs) and transfected into HepG2 cells as described above. Gel electrophoresis prior to transfection confirmed the successful complete ligation of the HBV DNA into cccDNA. The cells were washed and the growth medium replaced at 24 hours after transfection. Supernatant was then harvested at 48 hours for examination of protein production by ELISA.

Results

CpG islands in Hepatitis B DNA

The same three CpG islands identified previously by this laboratory [7] were again identified in the cloned virus using Methprimer (http://www.urogene.org/methprimer/index1.html): island 1, nucleotide positions 55-286; island 2, 1224 - 1667; and island 3, 2257 - 2443. The *CpG methyltransferase* used for in vitro methylation of the transfected HBV DNA targets all CpG dinucleotides. A total of 102 CpG dinucleotides were present, of which 63 (62%) were within the three predicted CpG islands and the remainder were scattered throughout the HBV genome. In contrast, *HpaII* methylase produces very low levels of methylation, with methylation targets present at only three locations in the cloned viral sequence: nucleotide positions 1156, 1571, and 2331.

Methylation of HBV DNA down-regulates viral mRNA production

Viral mRNA transcripts were produced when unmethylated HBV DNA sequences were transfected into HepG2 cells (Fig. 1A). However, mRNA transcripts detected with primers to the surface gene were almost undetectable when fully methylated DNA was transfected into HepG2 cells, $p = 0.018$ (Fig 1A). An intermediate but significant decrease in viral mRNA transcripts was evident when cells were transfected with HBV DNA with low levels of methylation, $p = 0.022$ (Fig. 1A). Diminished mRNA production was not a result of

reduced levels of closed circular HBV DNA, as in all cases the amount of closed circular forms were similar when measured by real time PCR (cells transfected with unmethylated HBV DNA: 6.92±0.07 log HBV cccDNA/mg total DNA; cells transfected with HpaII methylated HBV DNA: 6.86±0.05 log HBV cccDNA/mg total DNA; cells transfected with fully methylated HBV DNA: 6.82±0.07 log HBV cccDNA/mg total DNA).

While a decrease in transcripts was also observed using primers to the core gene, the decrease did not reach statistical significance (Figure 1A).

Methylation of HBV DNA reduces viral proteins in cell supernatants

ELISA was performed to study the effect of methylation on the accumulation of viral proteins in the supernatant. Abundant HBV surface and HBe proteins were detectable in the cell supernatant following transfection with unmethylated HBV DNA (Fig. 1B). In contrast, virtually no surface or e protein was detectable in the cell supernatants after transfection with fully methylated HBV DNA (Fig. 1B), $p < 0.001$. A significant decrease in secreted viral proteins was seen also seen when HepG2 cells were transfected with HBV DNA containing low levels of methylation (Fig. 1B), $p < 0.001$.

Methylation down-regulates HBV protein accumulation in hepatocytes

Immunohistochemistry was employed to understand the effect of methylation on cytoplasmic expression of viral proteins. Following transfection with unmethylated HBV DNA, approximately 10% of HepG2 cells showed strong cytoplasmic immunopositivity for HBsAg. Immunostaining for HBcAg showed rare positive cells, with less than 5% of cells showing nuclear staining. In contrast, when HepG2 cells were transfected with fully methylated HBV DNA, no HBsAg and no HBcAg were detectable by immunohistochemistry (Fig 2).

HBV cccDNA is methylated and methylation reduces viral protein production

Twelve liver samples from eight individuals were examined for methylation of cccDNA (Table 2). Heavy methylation was seen in one case, lower levels of methylation in nine cases, and no methylation in two cases. Methylation of cccDNA was seen in both tumor and non-tumor tissues.

To investigate whether methylation of cccDNA could impair protein production, cloned HBV DNA was released from the vector and circularized in vitro. The cccDNA then underwent in vitro methylation and was transfected into HepG2 cells. The wells with methylated cccDNA showed a greater than 90% reduction in HBsAg levels in the supernatant in comparison to wells transfected with non-methylated cccDNA ($p=0.01$).

Discussion

In chronic HBV infection, promoters, enhancers, and other cis-acting regulatory regions are known to have important roles in controlling HBV protein production and viral replication. In addition to these DNA encoded motifs, our data demonstrates a role for epigenetic changes. We have previously shown that HBV contains CpG islands [7,11]. The data presented in this study provides direct evidence that the CpG islands are functionally relevant to viral gene expression and demonstrate a novel mechanism for regulating viral protein production.

Methylation of human DNA is known to control expression of many human genes and these new findings suggest that viral DNA can interact with host transcription factors utilizing the same epigenetic mechanisms as normal host DNA. This finding is consistent with the

dependence of viruses, such as HBV, on host capabilities for viral survival. Thus, the presence of CpG islands and a role for epigenetic changes in the regulation of viral protein production likely reflects viral adaptation to host cells.

When HBV infects liver tissues but is non-integrated, the methylation density varies considerably [11]. In many cases, the methylation levels are low, with only few CpG dinucleotides methylated on any given DNA molecule. This variability in DNA methylation raises questions on the biological relevance of low levels of methylation. However, in this study we have shown that low levels of methylation can also effect viral protein production.

Regulation of HBV through epigenetic changes is further supported by previous studies in mice as well as human tissues. In mice with integrated tandem repeats of the complete HBV genome, no viral proteins were detectable until mice were treated with 5-azacytidine [12]. Others have reported similar results in separate animal models [13-15]. In human hepatocellular carcinomas with integrated HBV DNA, viral proteins were not expressed if the integrated HBV DNA was methylated [8].

In general, methylation of CpG islands works in conjunction with histone modifications to regulate gene expression. The hypothesis that epigenetic changes are important in regulating HBV infection is further strengthened by data showing that non-integrated HBV is associated with histones [16-18] and that the histones combine with HBcAg to form viral mini-chromosomes within the hepatocytes [18]. Furthermore, acetylation of histones can control HBV replication [16]. Together, these data provide compelling evidence that epigenetic factors contribute to the regulation of HBV protein production.

HBV DNA contains three predicted CpG islands but the precise role for each island is not clear. In the experiments reported here, targeting of individual CpG islands was not possible by in vitro methylation and the precise regions affected by methylation can only be surmised. Of the three HBV CpG islands, island 1 is in close proximity to the ATG start site of S gene, while island 2 overlaps with enhancers I and II as well as the core promoter. Island 3 contains the start codon for the P gene. We have previously shown that Hep3B cells are densely methylated on CpG island 1 [7]. Since Hep3B cells are known to produce hepatitis B surface protein, methylation of island 1 is an unlikely candidate for regulation of surface gene expression. Furthermore, we have previously shown that island 2 is more likely to be methylated in those samples with absent or very low levels of HBsAg production, suggesting island 2 may be more relevant to the surface gene [11]. It may be that methylation interferes with the enhancer II, which is known to regulate HBsAg production [2]. However, the data on these questions are limited and further studies will be required to precisely define the role for each of the CpG islands. In addition, while its true that CpG islands are the most likely candidates for gene regulation given the current understanding of DNA methylation, it remains possible that CpG dinucleotides outside of the predicted islands may also be involved in regulation of gene expression.

To our knowledge this study is the first to describe an assay for specifically identifying methylated HBV cccDNA. This assay exploits unique aspects of the HBV DNA and targets a portion of HBV CpG island 2. With this assay we were able to show that cccDNA in human tissues can be methylated, an important observation given the central role for cccDNA in viral replication. Based on our cell culture data, methylation of cccDNA can regulate protein production, but further quantitative studies of human blood and tissues will be necessary to understand the relationship between cccDNA methylation and viral DNA and protein levels.

In conclusion, HBV mRNA and protein expression can be regulated in vitro by methylation of viral DNA and low densities of CpG methylation retain the ability to reduce viral protein

production. Methylation of cccDNA also affects protein production and methylated cccDNA can be found in human tissues.

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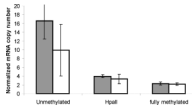

Figure 1A.

Real time PCR for surface/precore/pregenomic HBV transcripts (dark bars) and precore/pregenomic (light bars) transcripts. HBV mRNA is detectable in HepG2 cells harvested 48 hours after transfection with unmethylated HBV DNA. However, HBV DNA that has been fully methylated shows marked reduction in viral mRNA. Likewise, HBV DNA that has been methylated with *HpaII*, which leads to low levels of methylation, also shows decreased mRNA expression.

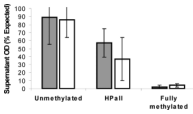

Figure 1B.

ELISA results for HBsAg (dark bars) and HBeAg (light bars) on cell supernatants harvested 48 hours after transfection with HBV DNA. Wells transfected with fully methylated HBV DNA show almost no proteins in the supernatants. Wells transfected with HBV DNA containing low levels of methylation (with *HpaII*) also show a significant decrease in proteins levels.

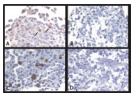

Figure 2.
Immunohistochemistry for HBcAg (panels A, B) and HBsAg (panels C, D) in HepG2 cells harvested 48 hours after transfection with HBV DNA. After transfection with unmethylated HBV DNA, HBcAg is detectable in the nuclei of a small proportion of HepG2 cells (panel A, arrows show examples of positive cells) but no HBcAg is detected after transfection with fully methylated HBV DNA (panel B). Likewise, HBsAg is detectable after transfection with unmethylated HBV DNA (panel C) but not with fully methylated DNA (panel D). All images are at 160X original magnification.

Table 1

PCR primers for HBV amplification.

Target	Primers	Product size
*HBV S gene[19]	Sense, For4, 5'- CCTATGGGAGTGGGCCTCA-3'	122
	Anti sense, Rev 7, 5'-CCCCAATACCACATCATCCATATA-3'	
cccHBV DNA[20]	cccDNA F'-5'-ACTCTTGGACTCBCAGCAATG-3'	260
	cccDNA R- 5'- CTTTATACGGGTCAATGTCCA-3'	
*HBV Core gene[21]	Sense COR 5'-GACCACCAAATGCCCCTAT -3'	
	Antisense COR 5'-CGAGATTGAGATCTTCTGCGA C-3'	139
HBV meth cccDNA	cccMeth F1 5'-TGGTTGTTAGGTTGTATTGTTAATTG-3'	300
	cccMeth R1 5'-AAAATCCAAAAATCCTCTTATATAAAACC-3'	

*Both of these primer sets can amplify pregenomic and precore transcripts because of overlapping reading frames within the HBV genome. The S gene assay will also detect S gene transcripts.

Table 2

Methylation of HBV cccDNA in human liver samples.

Sample No.	Age/gender/type of tissue[a]	HBV DNA copy number[c]	HBV genotype	No. clones analyzed	Methylation	
					No. of CpG's sites methylated	Total No. CpG dinucleotides methylated
1	59/F /N	3.5	C	3	0	0
2	59/F /T	3.5		5	2	8
3	37/M/N	2.5	A	NA[b]	NA	NA
4	37/M/T	3.4		5	0	0
5	65/M/N	5.8	A	5	1	1
6	65/M/T	5.3		6	28	99
7	76/M/N	2.6	A	4	1	1
8	76/M/T	3.5		7	7	7
9	74/F/N	1.8	A	5	3	9
10	57/M/T	7.3	A	8	5	8
11	66/M/T	5.8	A	7	1	1
12	23/F/T	4.9	A	7	2	2

[a] N, non-neoplastic tissue; T, hepatocellular carcinoma

[b] NA, Template did not amplify

[c] Log 10 HBV copy number per microgram of liver DNA