Methylation Regulates Hepatitis B Viral Protein Expression

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Background. Hepatitis B virus (HBV) DNA has been shown to contain CpG islands that are methylated in human tissue, which suggests a role for methylation in regulating viral protein production. However, data are lacking about 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 was transfected into HepG2 cells. In addition, a new assay was designed that specifically identifies methylated covalently closed circular DNA (cccDNA) in human liver tissue.

Results. Transfection of methylated HBV DNA led to reduced HBV mRNA levels in HepG2 cells, decreased surface and core protein expression in these 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 tumor and nonneoplastic human liver tissues. Finally, an in vitro equivalent of cccDNA showed decreased viral protein production in HepG2 cells after DNA methylation.

Conclusion. Taken together, these data demonstrate that methylation of viral CpG islands can regulate viral protein production.

More than 350 million persons are estimated to have chronic hepatitis B virus (HBV) infection, which can be identified by the persistent presence of the HBV surface antigen (HBsAg) in the blood [1]. The expression of viral proteins in host tissue is necessary for viral replication and the development of persistent infection. HBV protein expression is regulated by both cis-acting elements encoded in the HBV genome and by various trans-acting host factors. The cis-acting mechanisms include promoters for the precore and pregeneric transcripts, the preS1 transcript, the preS2/S transcript, and the X transcript. HBV DNA also contains 2 enhancers [2], as well as a negative regulatory region [3]. These regulatory regions can operate both independently and in cooperation with each other to regulate viral protein production and viral replication. For example, the tissue specificity of HBV replication is in part determined by the combined effects of the core promoter and 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 mechanisms similar to those by 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 the regulation of HBV gene expression.

In human tissue, 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 gene expression is unknown. HBV DNA can be methylated in human tissue in both unintegrated form [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 the present study, we demonstrate that methylation of viral DNA regulates viral gene expression and that methylated HBV covalently closed circular DNA (cccDNA) is present in human tissues.

METHODS

Cell culture system. We used the system described by Gunther et al. [9] as a model of HBV infection. In brief,
a cloned HBV genome is released from the vector and transfected into HepG2 cells as a 3.2-kb linear DNA fragment that contains 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 positive for hepatitis B e antigen (HBeAg) and HBsAg, and the mean serum HBV DNA level was 7.4 log_{10} copies/mL. Sequence analysis demonstrated that the virus was genotype D. Nucleotides were numbered from the EcoRI digestion site.

**Methylation of HBV DNA.** The cloned virus was digested with SapI 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.SslI; New England Biolabs). This enzyme methylates the cytosine in all CpG dinucleotides. Successful methylation was confirmed by using methylation-sensitive restriction enzymes that cut only unmethylated DNA (HhaI and HpaII; New England Biolabs). The unmethylated DNA used as a control was completely digested, whereas no digestion was observed for the methylated DNA. The integrity of the methylated DNA was confirmed by gel electrophoresis and by full-length polymerase chain reaction (PCR) amplification of the methylated DNA. In addition, the methylation status of islands 1 and 2 following transfection were investigated by use of bisulfite sequencing and cloning of the HBV DNA at 48 h after transfection. No loss of methylation was observed (data not shown).

The density of methylation in HBV CpG islands can range from low to high [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_{10} cells/well in standard 24-well plates and grown overnight in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and transfected (Lipofectamine 2000; Invitrogen) with 10 log_{10}-12 log_{10} 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 h. Each transfection experiment that compared unmethylated to methylated HBV DNA was performed in duplicate wells with ≥3 independent experimental replications.

The input concentration of HBV DNA (copies per reaction) was measured by real-time PCR prior to transfection. In control experiments, transfection with different numbers of unmethylated HBV DNA copies showed a linear relationship between the amount of DNA input and HBsAg and HBeAg production in the supernatant over the input range of ∼9–12 log_{10} HBV genome equivalents/well (R^2, 0.91 for HBsAg and 0.74 for HBeAg). Levels >12 log_{10} copies/well of HBV DNA appeared to saturate the system, whereas levels <9 log_{10} copies/well gave low and variable results. Thus, all experiments were performed by using HBV DNA input within the linear range, and findings were normalized for the amount of DNA input. By 2-way analysis of variance, there was no statistically significant difference between the groups regarding the average amount of transfected DNA (P = .1), with average inputs of 10.17, 10.51, and 10.64 log_{10} copies HBV DNA/well for the unmethylated, fully methylated, and partially methylated groups, respectively.

**HBV mRNA detection.** For the mRNA studies, 3 wells were used for each experimental condition. Total RNA was extracted from cells by using TRIzol (Invitrogen), followed by precipitation with isopropyl alcohol, in accordance with the manufacturer’s protocol. RNA extracts were treated with DNase I (Invitrogen), and cDNA was synthesized with oligo-dT primers by 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 reverse transcriptase. However, in experiments that used unmethylated HBV DNA, the mRNA signal was at least 100 times greater than the residual DNA signal, indicating that residual DNA contributed <1% to the quantification of the HBV mRNA.

Real-time PCR was performed with the SmartCycler System (Cepheid) by use of FastStart 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 as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 20 s, 55°C for 25 s, and 72°C for 25 s. Specificity was confirmed by melt-curve analysis and band size in 1% agarose gels. Primers directed against the core and surface HBV genome, both assays measure the combination of their target and other overlapping transcripts, such as the pregenomic transcript (table 1).

mRNA levels were quantitated by using an absolute standard curve. Data was normalized to the amount of HBV cccDNA 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 performed with 100 µL of supernatant, in accordance with the manufacturer’s instructions. In one set of experiments, cells were fixed in formalin, embedded in paraffin, and subjected to rou-
time immunohistochemical analysis for HBsAg and HBV core antigen (HBCAg). Antibodies (Dako) were used at 1:25 dilution and 1:900 dilutions, respectively. Student’s t tests were used to test differences in mRNA and protein expression among groups.

Studies of HBV cccDNA. We also sought to determine whether cccDNA was methylated in human liver tissue. Primers for bisulfite sequencing were designed to target a portion of island 2 and to be specific 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 we designed bisulfite sequencing primers that amplified only the positive strand of DNA. Because 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 DNA in all cases. Twelve liver tissue samples from 8 individuals with chronic HBV infection were then studied by using the cccDNA bisulfite sequencing primers. The use of human tissue was preapproved by the institutional review board. All samples were shown to be negative for HBV DNA integration by use of the Alu-HBV PCR assay [13]. DNA was extracted from 20–25 mg of liver tissue (QIAamp DNA Mini Kit; QIagen) and bisulfite treated (EpiTect Bisulfite Kit; QIagen), amplified with cccDNA-specific primers (table 1), and cloned. There were 5–8 clones submitted for sequencing.

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 (M.SssI; New England Biolabs) and transfected into HepG2 cells, as described above. Gel electrophoresis prior to transfection confirmed successful complete ligation of the HBV DNA into cccDNA. The cells were washed, and the growth medium was replaced 24 h after transfection. Supernatant was then harvested at 48 h for examination of protein production by use of ELISA.

RESULTS

CpG islands in HBV DNA. The same 3 CpG islands identified previously by this laboratory [7] were again identified in the cloned virus by use of Methprimer (http://www.urogene.org/methprimer/index1.html); the islands were as follows: 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 3 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 3 locations in the cloned viral sequence: nucleotide positions 1156, 1571, and 2331.

Methylation of HBV DNA and down-regulation of viral mRNA production. Viral mRNA transcripts were produced when unmethylated HBV DNA sequences were transfected into HepG2 cells (figure 1A). However, mRNA transcripts detected with primers for the surface gene were almost undetectable when fully methylated DNA was transfected into HepG2 cells (P = .018) (figure 1A). An intermediate but statistically significant decrease in the mean number of viral mRNA transcripts was evident when cells were transfected with HBV DNA with low levels of methylation (P = .022) (figure 1A). Diminished mRNA pro-

Table 1. Polymerase chain reaction primers used for hepatitis B virus (HBV) DNA amplification.

<table>
<thead>
<tr>
<th>Target, primers</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV S gene[a] [10]</td>
<td>122</td>
</tr>
<tr>
<td>Sense, For 4, 5'-CCTATGGGAGTGGGCCTCA-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense, Rev 7, 5'-CCCCAATACCACATCATCCATATA-3'</td>
<td></td>
</tr>
<tr>
<td>cccDNA F-5'-ACTCTTGGAGCTBCAGCAATG-3'</td>
<td></td>
</tr>
<tr>
<td>cccDNA R- 5'-CTTTTATACGGTGATCTGCA-3'</td>
<td></td>
</tr>
<tr>
<td>HBV core gene[a] [12]</td>
<td>139</td>
</tr>
<tr>
<td>Sense COR 5'-GACCACCAAAATGCCCTAT-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense COR 5'-CGAGATTGAGATCTTCTGCGAC-3'</td>
<td></td>
</tr>
<tr>
<td>Methylated HBV cccDNA</td>
<td>300</td>
</tr>
<tr>
<td>cccMeth F1 5'-TGTTGTATGGGTATGTGTTAATTG-3'</td>
<td></td>
</tr>
<tr>
<td>cccMeth R1 5'-AAAATCCAAAAATCTCTATATGAAACC-3'</td>
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</tbody>
</table>

NOTE. cccDNA, covalently closed circular DNA.

[a] Both of these primer sets can amplify pregenomic and precore transcripts because of overlapping reading frames in the HBV genome. The S gene assay also detects S gene transcripts.
Methylation of HBV DNA and reduction of viral protein production. Twelve liver tissue samples from 8 individuals were examined for methylation of cccDNA (table 2). High levels of methylation were seen in 1 sample, lower levels of methylation were seen in 9 samples, and no methylation was seen in 2 samples. Methylation of cccDNA was seen in both tumor and non-neoplastic 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 containing cells transfected with fully methylated cccDNA showed >90% reduction in HBsAg levels in the supernatant, compared with wells of cells transfected with unmethylated cccDNA (P = .01).

DISCUSSION

In individuals with chronic HBV infection, promoters, enhancers, and other cis-acting regulatory regions are known to play important roles in controlling HBV protein production and viral replication. In addition to these DNA-encoded motifs, our data demonstrate a role for epigenetic changes. We have previously shown that HBV contains CpG islands [7, 14]. The data presented in this study provide 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 the expression of many human genes, and these new findings suggest that viral DNA can interact with host transcription factors by using the same epigenetic mechanisms as normal host DNA. This finding is consistent with the dependence of viruses such as HBV on host capabilities for 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 tissue but is nonintegrated, methylation density varies considerably [14]. In many cases, methylation levels are low, and only few CpG dinucleotides are methylated on any given DNA molecule. This variability in DNA methylation raises questions about the biological relevance of low levels of methylation. However, in this study we have shown that low levels of methylation can also affect viral protein production.

The regulation of HBV through epigenetic changes is further supported by previous studies in mice as well as studies that involved human tissue. In mice with integrated tandem repeats of the complete HBV genome, no viral proteins were detectable until mice were treated with 5-azacytidine (demethylation agent) [15]. Others have reported similar results in different animal models [16–18]. In tissue from human hepatocellular car-

Table 2. Methylation of hepatitis B virus (HBV) covalently closed circular DNA in human liver samples.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Age of subject, years</th>
<th>Sex of subject</th>
<th>Type of tissue</th>
<th>HBV DNA, log_{10} copy number/mg total DNA</th>
<th>HBV genotype</th>
<th>Clones analyzed, no.</th>
<th>CpG sites methylated, no.</th>
<th>CpG dinucleotides methylated, no.</th>
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<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>N</td>
<td>3.5</td>
<td>C</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>59</td>
<td>F</td>
<td>T</td>
<td>3.5</td>
<td>C</td>
<td>5</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>M</td>
<td>T</td>
<td>3.4</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>M</td>
<td>N</td>
<td>5.8</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>M</td>
<td>T</td>
<td>5.3</td>
<td>A</td>
<td>6</td>
<td>28</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>M</td>
<td>N</td>
<td>2.6</td>
<td>A</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>76</td>
<td>M</td>
<td>T</td>
<td>3.5</td>
<td>A</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>74</td>
<td>F</td>
<td>N</td>
<td>1.8</td>
<td>A</td>
<td>5</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>57</td>
<td>M</td>
<td>T</td>
<td>7.3</td>
<td>A</td>
<td>8</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>66</td>
<td>M</td>
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<td>5.8</td>
<td>A</td>
<td>7</td>
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<td>1</td>
</tr>
<tr>
<td>12</td>
<td>23</td>
<td>F</td>
<td>T</td>
<td>4.9</td>
<td>A</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE. N, nonneoplastic tissue; NA, template did not amplify; T, tissue from hepatocellular carcinoma.
cinomas with integrated HBV DNA, viral proteins were not expressed if the integrated HBV DNA was methylated [8].

In general, the methylation of CpG islands works in conjunction with histone modifications to regulate gene expression. The hypothesis that epigenetic changes are important in the regulation of HBV infection is further strengthened by data showing that nonintegrated HBV is associated with histones [19–21] and that the histones combine with HBCAg to form viral minichromosomes in the hepatocytes [21]. Furthermore, acetylation of histones can control HBV replication [19]. Together, these data provide compelling evidence that epigenetic factors contribute to the regulation of HBV protein production.

HBV DNA contains 3 predicted CpG islands, but the precise role for each island is not clear. In the experiments reported here, it was not possible to target individual CpG islands by in vitro methylation, and the precise regions affected by methylation can only be surmised. Of the 3 HBV CpG islands, island 1 is in close proximity to the ATG start site of the S gene, whereas 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]. Because 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 samples in which HBsAg production is absent or present at very low levels, which suggests that island 2 may be more relevant to the regulation of the surface gene [14]. It may be that methylation interferes with 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 of 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 specifically designed to identify methylated HBV cccDNA. This assay exploits unique aspects of HBV DNA and targets a portion of HBV CpG island 2. By use of this assay, we were able to show that cccDNA in human tissue can be methylated, which is an important observation given the central role of cccDNA in viral replication. Our cell culture data indicate that methylation of cccDNA can regulate protein production, but further quantitative studies of human blood and tissue 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 tissue.

References