Integrated hepatitis B virus DNA sequences specifying the major viral core polypeptide are methylated in PLC/PRF/5 cells

(Rpa II and Msp I; gene expression/hepatitis B surface antigen/Southern blot analysis/hepatocellular carcinoma)

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ABSTRACT  The methylation of various hepatitis B virus (HBV) DNA sequences was examined using the restriction endonucleases Rpa II and Msp I. HBV DNA from virions (Dane particles) and virus-infected liver tissue was digested with Rpa II or Msp I and fractionated by electrophoresis in agarose gels, and the restriction enzyme cleavage pattern was examined by Southern blot analysis. No methylation of the 5' C-C-G-G 3' recognition sequence was detected in either virion DNA or HBV DNA from infected liver tissue. The tissue culture cell line PLC/PRF/5, derived from a human hepatoma, possesses HBV DNA exclusively integrated at a site that DNA with Rpa II and Msp I revealed that the integrated HBV DNA sequences were methylated. Further analysis using probes specific for various regions of the HBV genome showed that some of the hepatitis B viral DNA sequences, including those specifying the major surface antigen polypeptide, were methylated infrequently or not at all. In contrast, the viral DNA sequences coding for the major core polypeptide were extensively methylated. Because the surface antigen is expressed in these cells while the core antigen is not, our results suggest that DNA methylation could account for the selective expression of HBV genes in this hepatoma cell line.

The development of hepatocellular carcinoma in man has been shown to be strongly associated with chronic hepatitis B virus (HBV) infection in many parts of the world (1, 2). The sequence of the entire 3.2-kilobase-pair (kb) genome of HBV has been determined and the coding sequences for the major polypeptides of the viral core (HBcAg) and surface antigen (HBsAg) have been localized (3-5). HBV DNA has been found to be integrated in the cellular DNA of primary hepatomas (6-8) and hepatoma cell lines (9-13). The tissue culture cell line PLC/PRF/5, isolated from a human hepatoma, synthesizes HBsAg but not HBcAg and whole virions (9, 14, 15). The reason for the selective expression of the HBsAg gene is not known.

Evidence is accumulating that methylation of DNA may play a role in gene expression. The presence of methylated DNA sequences can be determined by restriction enzyme cleavage of the DNA. One of the most useful systems is the digestion of DNA with Msp I and Rpa II. Although both enzymes recognize the DNA sequence 5' C-C-G-G 3', Rpa II will not cleave the DNA if the internal cytosine is methylated. Several groups of investigators, using restriction endonucleases, have shown that active genes in herpes simplex virus (16), mouse mammary tumor virus (17, 18), and higher eukaryotes (19) are not methylated. As a general rule, it appears that absence or low levels of methylation of DNA correlates with active transcription of a gene. Conversely, several groups have shown that in vitro methylation of a normally active gene inhibits gene expression. Among those studied are the herpes simplex virus thymidine kinase gene (20), the hamster adenine phosphoribosyltransferase gene (21), and the early region of the adenovirus genome (22). In agreement with these studies, several groups have shown that demethylation of an inactive methylated gene restores gene activity. Addition of the cytidine analogue 5-azacytidine to dividing cells prevents the methylation of newly synthesized DNA. Clough et al. (23) have shown that such treatment of cultured mouse cells containing an inactive methylated herpes simplex virus thymidine kinase gene restores active gene expression. This is supported by other reports that demethylation is accompanied by an increase in gene expression (24, 25) and will restore the infectivity of a methylated endogenous murine leukemia virus (26).

This paper presents data indicating that HBV DNA in virions and in virus-infected tissue is not methylated. HBV DNA integrated in the DNA of PLC/PRF/5 cells, however, is methylated. Identification of the methylated DNA sequences showed that HBsAg DNA sequences are only slightly methylated but HBcAg DNA sequences are extensively methylated. Methylation of the HBcAg DNA sequences could account for the lack of expression of this gene in PLC/PRF/5 cells.

MATERIALS AND METHODS

Isolation of DNA. HBV DNA was isolated from virion-containing plasma (HBsAg subtype adw) as described (27). The single-stranded region of the HBV DNA was made double stranded by the endogenous virion DNA polymerase and by avian myeloblastosis virus reverse transcriptase (28). Liver obtained at autopsy from a HBsAg carrier was the source of intracellular HBV DNA. Nuclei were prepared by homogenization of hepatic tissue in 10 mM Tris-HCl, pH 7.5/0.3 M NaCl/10 mM EDTA and centrifugation at 500 × g (5 min, 4°C). The nuclei were washed and pelleted twice in the above solution and once in 10 mM Tris-HCl, pH 7.5/0.3 M NaCl/10 mM EDTA/0.2% Triton X-100. The DNA from nuclear and cytoplasmic fractions was obtained by proteinase K digestion in 50 mM Tris-HCl, pH 7.5/1 mM EDTA/1% NaDodSO4 (100 μg/ml, 2 hr, 60°C) followed by phenol/chloroform extraction and ethanol precipitation. PLC/PRF/5 cells, grown in culture as described (9), were lysed in 50 mM Tris-HCl, pH 7.5/1 mM EDTA/1% NaDodSO4, and the DNA was obtained as described above. RNA was removed by digestion with pancreatic RNase A (25 μg/ml, 16 hr, 25°C).

Enzyme Digestion, Radiolabeling of Probe, and Modified Southern Blot Analysis. Restriction endonuclease (EcoRI, HindIII, Msp I, and Rpa II) digestions were carried out using conditions recommended by the manufacturer (New England BioLabs). The enzyme digestions were monitored by adding

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; kb, kilobase pair(s).

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recombinant HBV DNA to parallel reaction mixtures to confirm complete digestion of the DNA. DNA fragments were separated by electrophoresis in 0.5–2.0% agarose gels (25×25 cm bed) and the DNA was transferred to diazotized aminothiophenol paper (29, 30). The recombinant DNA probes, the entire HBV genome (31) and a viral DNA fragment containing the HBsAg coding sequence in pBR322 (32), have been described. The DNA probes for other regions of the HBV genome (see Fig. 4) were obtained by Msp I digestion of a pBR322–HBV recombinant, separation of the DNA fragments by agarose gel electrophoresis, and elution of specific HBV DNA fragments by the method of Dretzen et al. (33). The various HBV DNA probes were nick-translated (34) to high specific activity (2–5 × 10^5 cpm/μg) using one or two [32P]dNTPs (Amersham; 3,000 Ci/mmol; 1 Ci = 37 GBq). Hybridization conditions were those described by Davis et al. (35).

RESULTS

Methylation of HBV DNA from Virions and Virus-Infected Tissue. An effective method for determining whether DNA is methylated involves the use of the restriction enzymes Hpa II and Msp I. Both enzymes recognize the DNA sequence 5' C-C-G-G 3', but Hpa II will not cleave that sequence when the internal cytosine is methylated. When equal amounts of DNA are digested with the two enzymes, any difference in the cleavage pattern indicates the presence of a methylated cytosine. Since HBV has not yet been propagated in tissue culture, virus DNA was isolated from hepatitis B virions (HBsAg subtype adw) concentrated from the plasma of a HBsAg carrier (36). The restriction enzyme cleavage patterns of HBV DNA digested with HindIII, EcoRI, Msp I, and Hpa II are shown in Fig. 1. After cleavage, the DNA fragments were fractionated by agarose gel electrophoresis, transferred to diazotized aminothiophenol paper, hybridized with a recombinant HBV–pBR322 [32P]DNA probe containing the entire (3.2-kb) HBV genome, and examined by autoradiography. The results indicate that the HBV DNA was not cut by HindIII (lane d), which has been the case for all HBV DNAs thus far examined (28, 31). EcoRI, which cleaves the DNA of most HBV subtypes once (28) produced two DNA fragments (lane c), indicating the presence of two EcoRI sites in the DNA of this HBV isolate. The Hpa II and Msp I enzyme digests were identical (lanes a and b, respectively), indicating the absence of methylation in the recognition sequence for these enzymes. The DNA bands—2.1, 0.8, and 0.3 kb—found after Hpa II and Msp I digestion were those expected for double-stranded DNA from HBV of subtype adw (32). Thus, no methylation could be detected in virion DNA examined in this way.

To examine the DNA of HBV-infected cells for methylation, DNA was isolated from nuclear and cytoplasmic fractions of liver tissue of two different HBV-infected patients and examined by Hpa II and Msp I digestion and Southern blot analysis. The results for one of the liver DNAs are shown in Fig. 2. There was no detectable difference in the cleavage patterns of the DNAs of either the nuclear or the cytoplasmic preparation. Thus, HBV DNA in the nuclei and cytoplasm of these liver cells is apparently not methylated at the 5' C-C-G-G 3' sites. Comparison of the restriction enzyme cleavage patterns indicated that the nuclear (lanes a and b) and cytoplasmic (lanes c and d) DNA preparations contained different viral DNA forms. The HBV DNAs in the cytoplasmic preparations were electrophoretically more heterogeneous, indicating the presence of single-stranded regions (37) that were larger and more variable in length than those in the DNAs of the nuclear preparations.

HBV DNA Integration Sites in PLC/PRF/5 DNA. Evidence has been obtained that PLC/PRF/5 cells derived from human hepatoma tissue contain HBV DNA sequences exclusively in an integrated state (9–13) and the only viral gene expressed in these cells appears to be that specifying HBsAg. Integration of HBV DNA was suggested by HindIII digestion of PLC/PRF/5 DNA and Southern blot analysis with a HBV [32P]DNA probe. Because HindIII does not cleave HBV DNA, integrated viral DNA sequences would be expected to be found in DNA fragments larger than unit-length (3.2-kb) HBV DNA and with cellular DNA sequences at both ends after digestion of PLC/PRF/5 DNA with HindIII. We have carried out such experiments by fractionating HindIII-digested PLC/PRF/5 DNA by electrophoresis in long-bed (25×25 cm) 0.5–1.0% agarose gels, transferring the DNA to diazotized aminothiophenol paper, and hybridizing with HBV–pBR322 [32P]DNA probes. Autoradiograms showed that, in addition to the previously reported (9–13) HBV DNA-containing bands, several other bands had been resolved. The results of an experiment in which at

![Fig. 1. Southern blot analysis of HBV DNA isolated from Dane particles. Single-stranded regions of HBV DNA were made double stranded by an endogenous virion DNA polymerase reaction followed by an avian myeloblastosis virus reverse transcriptase reaction with the DNA isolated from virions. The DNA was digested with Hpa II (lane a), Msp I (lane b), EcoRI (lane c), or HindIII (lane d) or was not digested (lane e) and then fractionated by electrophoresis in a 1.5% agarose gel. The DNA was transferred to diazotized aminothiophenol paper and hybridized with a HBV [32P]DNA probe.](image-url)
least eight HBV DNA-containing bands having positions expected for linear fragments of >40, 39, 24, 17.2, 11.7, 6.6, 4.8, and 1.9 kb are shown in Fig. 3A. All but the smallest of these bands are larger than unit-length HBV DNA. No HBV DNA-containing bands were detected in uncleaved cell DNA. These HBV DNA-containing fragments did not result from incomplete enzyme digestion because exogenous recombinant HBV DNA was completely digested in parallel reactions. The additional bands were not a recently acquired feature of PLC/PRF/5 DNA due to DNA rearrangements; comparison of frozen PLC/PRF/5 cells of early passage (1978) with cells continuously passaged since that time revealed no differences in DNA fragments containing viral sequences after HindIII digestion of the cell DNA. We conclude that HBV is integrated into at least eight sites on PLC/PRF/5 DNA.

HBV DNA Sequences Integrated in the DNA of PLC/PRF/5 Cells Are Methylated. PLC/PRF/5 DNA was digested with Msp I and Hpa II, and the restriction enzyme cleavage fragments were examined by agarose gel electrophoresis and Southern blot analysis (Fig. 3B). Identical viral DNA-containing fragments of 8.5, 3.0, 2.0, 1.8, 1.5, and 1.3 kb were found in cell DNA digested with either Msp I or Hpa II, indicating that the recognition sequences for Msp I were also cleaved by Hpa II and thus were not methylated. The Msp I DNA fragments of 1.6, 1.4, 1.1, and 0.5 kb and smaller had no corresponding Hpa II DNA fragments, indicating that the recognition sequence for this enzyme in these fragments was methylated. This methylation pattern was found to be stable for at least 30 cell generations (unpublished data). The HBV-specific Msp I DNA fragments in PLC/PRF/5 cell DNA do not correspond in size to those expected from intact virus (Fig. 1). This is most likely the result of rearrangement of viral gene sequences and endonuclease cleavage of flanking cellular DNA sequences. Therefore, to identify the regions of the HBV genome contained in the various restriction enzyme cleavage fragments of PLC/PRF/5 DNA, the HBV DNA probes described in Fig. 4 were used. Probe 1 contains the entire coding sequence for the major HBsAg polypeptide. Probe 2 contains DNA sequences between the coding sequences for the HBsAg and the HBeAg polypeptides. The viral DNA sequence in probe 3 contained almost all of the coding sequence for the major polypeptide of HBeAg particles.

Hpa II- and Msp I-digested PLC/PRF/5 DNA was fractionated by electrophoresis in 1 to 2% agarose gels and then hybridized to the three HBV [32P]DNA probes. An autoradiogram of a 2% agarose gel that resolves the lower molecular weight DNA bands of interest is shown in Fig. 5. DNA was hybridized to total HBV [32P]DNA (lanes a and b), to probe 1...
cleavage at and long viral DNA strands, respectively, and the positions of the 5' and 3' ends are shown. The regions of the HBV genome that were nick-translated and used as probes to identify the HBV DNA sequences in PLC/PRF/5 DNA are as follows: probe 1, previously described by Siddiqui et al. (32), represented a recombinant molecule containing plasmid pBR322 and a viral DNA fragment between Tac I (T) and Hpa I (H) restriction sites; probes 2 and 3 were isolated by Msp I digestion of a recombinant containing the complete HBV genome. Probe 2 contains the HBV DNA sequences between the HBsAg polypeptide gene and the core polypeptide gene, and probe 3 contains the DNA sequences coding for the core polypeptide (HBcAg). HindIII- and EcoRI-digested PLC/PRF/5 cell DNA was subjected to Southern blot analysis using all three [32P]DNA probes. The results were in complete agreement with those of Edman et al. (13). Only the results for some of the DNA fragments are presented here. The HindIII fragment of >40 kb hybridized to all regions of the HBV genome, while the 39-kb fragment hybridized only with probe 1. The 1.9-kb fragment hybridized strongly to probe 3 but only weakly to probe 2.

![Figure 4](image)

**Fig. 4.** Restriction enzyme cleavage map of the HBV genome (HBsAg subtype adw). HBV DNA (3.2 kb) is portrayed as a linear molecule after cleavage at the single EcoRI (E) site (bottom line). Msp I (M) cleaves HBV DNA in the positions shown. Lines designated a and I represent the short and long viral DNA strands, respectively, and the positions of their 5' and 3' ends are shown. The regions of the HBV genome that were nick-translated and used as probes to identify the HBV DNA sequences in PLC/PRF/5 DNA are as follows: probe 1, previously described by Siddiqui et al. (32), represented a recombinant molecule containing plasmid pBR322 and a viral DNA fragment between Tac I (T) and Hpa I (H) restriction sites; probes 2 and 3 were isolated by Msp I digestion of a recombinant containing the complete HBV genome. Probe 2 contains the HBV DNA sequences between the HBsAg polypeptide gene and the core polypeptide gene, and probe 3 contains the DNA sequences coding for the core polypeptide (HBcAg). HindIII- and EcoRI-digested PLC/PRF/5 cell DNA was subjected to Southern blot analysis using all three [32P]DNA probes. The results were in complete agreement with those of Edman et al. (13). Only the results for some of the DNA fragments are presented here. The HindIII fragment of >40 kb hybridized to all regions of the HBV genome, while the 39-kb fragment hybridized only with probe 1. The 1.9-kb fragment hybridized strongly to probe 3 but only weakly to probe 2.

![Figure 5](image)

**Fig. 5.** Identification of the methylated HBV DNA sequences in PLC/PRF/5 cell DNA. PLC/PRF/5 cell DNA was digested with Hpa II (lanes a, c, e, and g) or Msp I (lanes b, d, f, and h) and fractionated by electrophoresis in a 2% agarose gel. Southern blot analyses used the following HBV [32P]DNA-containing probes: total HBV DNA, lanes a and b; probe 1, lanes c and d; probe 2, lanes e and f; and probe 3, lanes g and h (see Fig. 4).
Therefore, two of the eight Msp I sites appeared to be methylated. Probe 3, containing DNA sequences coding for the core polypeptide, hybridized to seven Msp I DNA fragments, only one of which had a corresponding Hpa II DNA fragment. Thus, six of seven Msp I sites appeared to be methylated. Clearly, the core polypeptide gene is more extensively methylated than the HBsAg polypeptide gene.

DISCUSSION

Our results indicate that HBV DNA isolated from virus and virus-infected cells is not methylated at 5’ C+C-G-G 3’ sites. In contrast, HBV DNA integrated in the DNA of PLC/PRF/5 cells is methylated, and the methylation pattern is stable for more than 30 cell generations. Analyses of simian virus 40 (38), herpes simplex (39, 40), vaccinia (39), herpes saimiri (41), and adenovirus (42) DNAs have shown that DNA isolated from virus particles is either slightly methylated or not methylated at all. However, the DNA of at least one of the viruses, herpes simplex, is transiently methylated during the course of productive infection (40). Methylation of specific virus genes in virus-transformed cell lines has been reported for herpes saimiri virus (41) and adenovirus (42). Thus, the data reported here are consistent with those published for other DNA viruses. The purpose of these experiments was to determine whether methylation of HBV DNA in PLC/PRF/5 cells correlates with gene expression. Although other factors, such as rearrangement of HBV sequences, may be involved, a direct correlation was found. HBsAg is produced by PLC/PRF/5 cells and the integrated HBsAg DNA sequences are poorly methylated. In contrast, HBeAg is not produced and the DNA sequences coding for the major core polypeptide are extensively methylated. Thus, methylation of integrated HBV DNA may account for the differential expression of these HBV genes.

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