



ORIGINAL PAPERS

HBV integrants of hepatocellular carcinoma cell lines contain an active enhancer

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Hepatitis B virus (HBV) infection is a major risk factor worldwide for the development of hepatocellular carcinoma (HCC). Integrated HBV DNA fragments, often highly rearranged, are frequently detected in HCC. In woodchuck, the viral enhancer plays a central role in hepatocarcinogenesis, but in humans the mechanism of HBV oncogenesis has not been established. In this study we investigated the status of the viral enhancer in two human HCC cell lines, Hep3B and PLC/PRF/5 each containing one or more integrated HBV DNA fragments. Active enhancer was defined by virtue of its protein occupancy as determined by genomic *in vivo* DMS footprinting. In PLC/PRF/5 cells, the HBV DNA was integrated in a cellular gene at chromosome 11q13, at a locus reported to be amplified in many tumors. We show here that in both cell lines, the integrated HBV DNA fragments contain an active enhancer-I. In particular, the occupation of the two previously defined basic enhancer elements, E and EP, was prominent. While in both cell lines the same protein binds to the EP elements, the E element, however, is occupied in a cell-line specific manner. In PLC/PRF/5 but not Hep3B, the prominent binding of an undefined protein was detected. Our data suggest that this protein is likely to be the fetoprotein transcription factor (FTF). The finding that enhancer sequences are conserved and functional in different cell lines suggests a selection pressure for their long-term maintenance. We therefore propose that the HBV enhancer-I might play a role in hepatocellular carcinogenesis. *Oncogene* (2001) 20, 6811–6819.

Keywords: HBV enhancer; *in vivo* footprinting; onco-DNA; fetoprotein transcription factor (FTF)

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers. Epidemiological and other studies have established a relationship between the hepatitis B virus (HBV) carrier-state and development of HCC. HCC develops many years after HBV infection, strongly suggesting that no viral product directly causes cancer and thus that HBV does not encode a genuine

transforming viral oncogene. Nevertheless, expression of viral proteins such as pX and PreS2/S has been associated with the development of HCC (reviewed in Butel *et al.*, 1996; Hildt and Hofschneider, 1998). Both proteins have been shown to coactivate a wide variety of cellular and viral promoters. The HBV pX protein either directly or indirectly modulates different signaling pathways that may give rise to cell growth and viability on one hand, and promote cell death on the other, depending on the experimental conditions. In addition, based on identification of pX targets and biochemical analysis, it has been speculated that pX might modulate the cellular DNA repair machinery, thereby enhancing mutagenesis and transformation (Butel *et al.*, 1996). Nevertheless, in HBV positive HCC the p53 form present is frequently wild-type (Greenblatt *et al.*, 1997). A number of studies suggested that pX functionally inactivates p53 via several different mechanisms (Doitsh and Shaul, 1999 and the references therein). Interestingly, in woodchucks although no role has been attributed to pX in HCC development, the p53 gene is by large wild-type (Rivkina *et al.*, 1996). It remained to be clarified how p53 is inactivated in these tumors.

Integrated HBV DNA was found in a majority of HBV positive HCC, however the role of the integrants in hepatocarcinogenesis is not known. In many tumors, the HBV integrants contain a C-terminal truncated pX open reading frame (ORF) due to the integration 'hot-spot' site in the HBV genome that is positioned at the end of the pX ORF. The truncated pX that often lacks the C-terminal 5–15 amino acids might be a gain of function mutant that promotes oncogenesis. In this regard, it is interesting to note that the truncated pX becomes predominantly nuclear (Chami *et al.*, 2000). Interestingly, truncated pX can be also generated by an alternative mechanism that employs a cryptic polyadenylation signal inside the pX ORF (Kairat *et al.*, 1999).

Insertional activation of cellular proto-oncogenes by integration of cis-acting HBV elements has also been proposed. In woodchuck, integrated active enhancer is a hallmark of WHV oncogenesis. Site specific integration of woodchuck hepatitis virus (WHV) DNA next to proto-oncogenes is common (Fourel *et al.*, 1994). In these cases, the WHV integrants contain active enhancers (Flajolet *et al.*, 1998) that are apparently essential in activating the nearby proto-oncogenes. However, the possibility that in these cases pX also contributes to oncogenesis was not ruled out. In

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humans, despite intensive searches, only few cases of HCC proved positive for proto-oncogene activation (Chami *et al.*, 2000 and the references therein). The presence of an enhancer region in many HBV integrants is well established, but whether it is functional is an open question.

The mode of HBV gene expression is unique in the sense that the synthesis of all the viral transcripts is regulated predominantly at the transcriptional level and not by RNA processing (in sharp contrast to retroviruses, for example). Every single nucleotide of the viral DNA is engaged in at least one ORF, leaving no 'coding-free' DNA segments exclusively devoted to regulation. Consequently, all the regulatory DNA cis elements, e.g. promoters, enhancers and the poly(A) signal, overlap with the existing ORFs. Furthermore, the regulatory cis-elements are sometimes bifunctional, as underscored by the fact that the deviated poly(A) box also has TATA-box function (Paran *et al.*, 2000). The viral genome contains multiple promoters whose activity is regulated mainly by enhancer-I and -II, the former positioned upstream, while the latter is inside the X ORF. This compact organization is the major reason for the difficulty in separating the contribution of the enhancers from pX in oncogenesis.

The PLC/PRF/5 and Hep3B HCC cell lines contain integrated HBV sequences. PLC/PRF/5 (Alexander) cells contain multiple HBV integrants and are active in HBsAg production. The integrated HBV DNA fragments have been cloned and analysed (Shaul *et al.*, 1984; Ziemer *et al.*, 1985). Seven different HBV integrants were identified and none contained an intact pX ORF. Previously, it was shown that one of the PLC/PRF/5 integrants is positioned next to a cellular gene that encodes a ribosomal protein (Ou *et al.*, 1987). It has been speculated that the enhanced expression of this gene in PLC/PRF/5 cells might be attributed to the viral sequences. However insertional activation might not be the only model for transformation of these cells, and in fact, others have reported integration of HBV DNA in the cellular mevalonate kinase gene in PLC/PRF/5 cells, resulting in the production of a chimeric preS2-mevalonate protein (Graef *et al.*, 1994).

One of the integrants, clone Al-26, contains a linear DNA sequence with an intact HBsAg gene. High production of HBsAg was obtained when CHO cells were transfected with this clone (unpublished observation). Production of HBsAg was also observed when SK-Hep1 cells were transiently transfected with clone Al-26. Furthermore, in that case removal of *StuI*-*SphI* HBV fragment that contains the enhancer sequence resulted in a sharp reduction in HBsAg synthesis (Faktor *et al.*, 1988).

Here, we show that the Al-26 integrant is located in a chromosomal region that is amplified in many tumors. By employing genomic DMS footprinting we show that these integrants contain active enhancer-I by virtue of their capacity to bind cellular DNA-binding proteins. Our data suggest that the integrated active HBV enhancer is preferentially maintained in HCC cells, and therefore might play a role in hepatocarcinogenesis.

Results

Structure of an HBV-DNA integrant of PLC/PRF/5 cells

Clone Al-26, an HBV integrant in PLC/PRF/5 cells, was sequenced and was found to contain an intact HBsAg gene including the sequences of the known preS1 and preS2 promoters and the enhancer-I (Figure 1). This integrant contains highly modified enhancer-II sequence and the pX ORF contains a stop codon after the first seven amino acids. Interestingly, this HBV sequence is integrated inside a cellular ORF between the coding and the promoter regions in an opposite orientation. The sequence of this ORF was reported (accession number HU79266), and based on *in silico* (bioinformatic) analysis, it might represent a nuclear protein involved in cell cycle regulation (data not shown). The intriguing possibility that this ORF has tumor suppressor function and that it is inactivated by the inserted HBV sequences deserves further study. This HBV integrant maps to the chromosomal region, 11q13 (Figure 1), a region amplified in many carcinomas (Shuster *et al.*, 2000 and references therein). We therefore investigated this integrant to determine whether its enhancer is active *in vivo*.

DMS footprinting of HBV enhancer-I

DMS footprinting detects the DNA G-residues that are in contact with the binding proteins. An occupied region is characterized by either lower or higher intensity of the bands cleaved at the G-residues, as compared with the corresponding naked DNA treated in a similar manner. Before performing genomic *in vivo* DMS footprinting, we analysed the HBV enhancer *in vitro* after incubation with PLC/PRF/5 cell nuclear extracts. To further analyse the protein binding regions, we employed HBV enhancer mutants at the sites of interest (Dikstein *et al.*, 1990b). When wt enhancer sequence was used as a probe, the G-residues at the E, NF1-binding site and EP regions were clearly detected (Figure 2a). When each of these binding sites were mutated, no G-residues were protected at the corresponding regions, suggesting that the interaction is specific and that this interaction is detectable by DMS footprinting.

The sequences of the integrated HBV enhancer of clone Al-26 of PLC/PRF/5 cells and the unique integrant of Hep3B cells were determined and compared to HBV adw2 subtype (Figure 2b). This enhancer sequence is highly conserved with minimal modifications. In sharp contrast, the enhancer II region was highly modified (Berger and Shaul, 1987).

Enhancer-I is occupied in vivo in PLC/PRF/5 cells

Having demonstrated that PLC/PRF/5 nuclear extracts occupy the HBV enhancer *in vitro* we next performed genomic DMS footprinting by the ligation-mediated polymerase chain reaction (LM-PCR) technique, to assess enhancer occupancy *in vivo*. To this end we synthesized a number of oligonucleotides primers

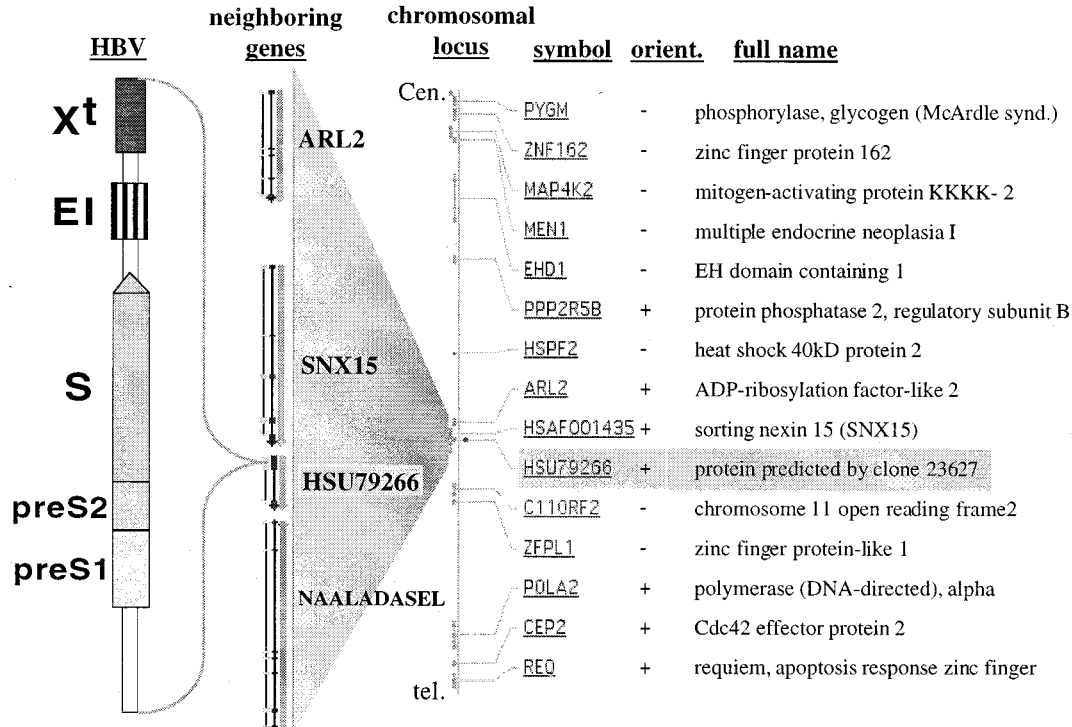


Figure 1 Schematic presentation of Al-26 clone integration in PLC/PRF/5 cells. The chromosomal locus of the integration site on chromosome 11q13.1-13.5 (NCBI Genome Map View) is shown. The neighboring genes to the integration site include SNX15, ARL2 and N-acetylated alpha-linked acidic dipeptidase-like (NAALADASEL). The integration site within the first exon of the predicted gene HSU79266, is shown in the opposite direction. The exons are presented as boxes. The HBV sequences that are present in this integrant, include the different HBsAg ORFs, enhancer-I and a truncated X ORF (X')

specific for clone Al-26. The analysis was performed on both the coding and the non-coding strands. The E element region was protected as was observed by the lower intensity of the G-residue at position 2600, and higher intensity bands at positions 2602 and 2596 (Figure 3, non-coding panel). This region was also protected when the coding strand was analysed. In that case, a specific reduction in the intensity of the G-residues at the positions 2598 and 2601, and to a lesser extent at position 2595 were seen (Figure 3 coding panel). Interestingly, these very same residues were protected when an *in vitro* assay was performed (Figure 2) suggesting that the same proteins may bind the enhancer region under both sets of conditions. However, in contrast to the *in vitro* analysis, the *in vivo* footprinting also detected DMS hyper-reactive G-residues, suggesting that *in vivo* the E element encountered unique interactions or modifications.

The EP element was also DMS protected. Residues 2571 and 2568 are slightly protected, whereas residues 2564 and 2563 are fully protected. On the coding strand, residues 2562, 2566 and 2567 were protected. In the autoradiogram shown, the 2559 G-residue is not detected although sequence analysis confirmed its presence in the Al-26 clone (Figure 2b). In general the genomic footprinting pattern of the EP element is similar to that of *in vitro* data (Figure 2). In addition significant protection is evident at the region between the EP and GB elements (2556–2558); however, the

nature of the binding protein is not known. Additional protected G-residues are located at the GB region in the non-coding strand, and at the HNF3-binding site at the coding strand. Interestingly, *in vivo* binding of a protein at the 5' region of the enhancer is clearly detected. This region was previously reported to be protected *in vitro* (Kosovsky and Siddiqui, 1999).

HBV enhancer-I is inactive in heterologous cells

Clone Al-26 was transfected into CHO cells and stable cell lines were established that expressed high levels of HBsAg (data not shown). These cells were subjected to DMS footprinting to examine the behavior of the HBV enhancer under a heterologous environment. In contrast to our observations in PLC/PRF/5 cells, no significant protection was detected (Figure 4). The fact that no binding of the ubiquitous transcription factors, such as RFX1, was observed was rather surprising and may suggest that a large repertoire of binding protein is needed to establish a functional enhancer.

HBV enhancer-I in Hep3B cells is active in vivo

Genomic DMS footprinting of the enhancer region of the integrated HBV sequence in Hep3B was performed. Both the coding and the non-coding DNA strands were analysed. At the non-coding strand, the G-residue 2600 of the E element was protected, whereas residue

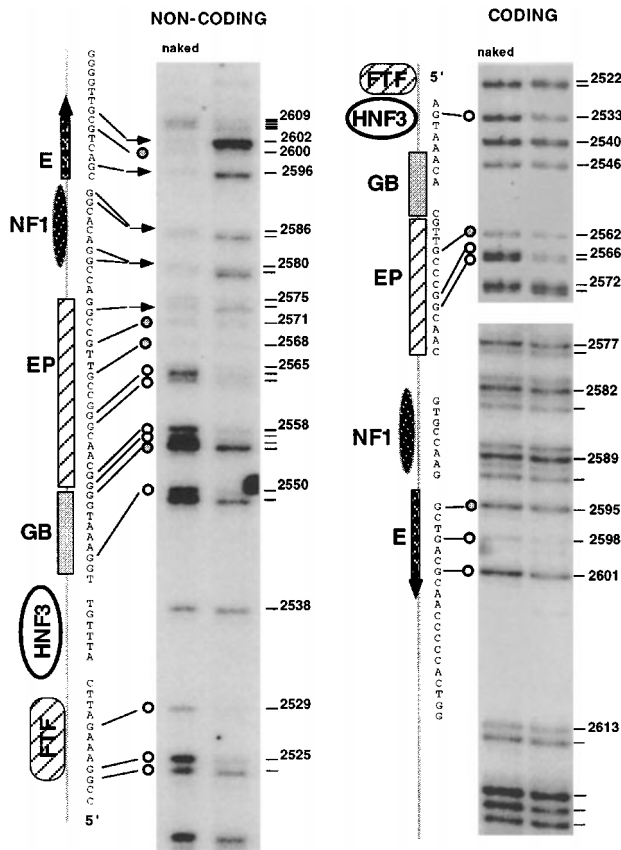


Figure 3 Genomic *in vivo* DMS footprinting of enhancer-I region of an HBV integrant in PLC/PRF/5 cells. PLC/PRF/5 cells were subjected to the DMS reaction and the DNA was extracted and processed as detailed in Methods. For the analysis of the non-coding strand, the primers 1-3 (UPI-3 see Materials and methods) were utilized whereas primers DP1-3 were employed for the analysis of the coding strand. In parallel, DNA was first extracted from PLC/PRF/5 cells and then subjected to the DMS reaction (naked lanes). The known HBV enhancer elements with their corresponding sequences and the G-residues that were either protected (open circles indicate full protection, and dotted circles indicate poor protection) or DMS hyper-reactive (arrows) are shown

(FTFm). The mutant reporter displayed much lower activity in PLC/PRF/5 and Huh7 hepatoma cell lines but not in Hep3B cells (Figure 6d). This finding is consistent with the lack of occupation of the FTF site in Hep3B (Figure 5). These results strongly suggest that the FTF or a related protein is a functional activator of the HBV enhancer-I in PLC/PRF/5 cells, and that Hep3B cells do not express the protein at functional levels.

Discussion

In this study, HCC cell lines containing integrated HBV were utilized to determine whether the viral enhancer is active *in vivo*. Since the hallmark of an active enhancer is its interaction with specific DNA binding proteins, we adopted genomic *in vivo* DMS

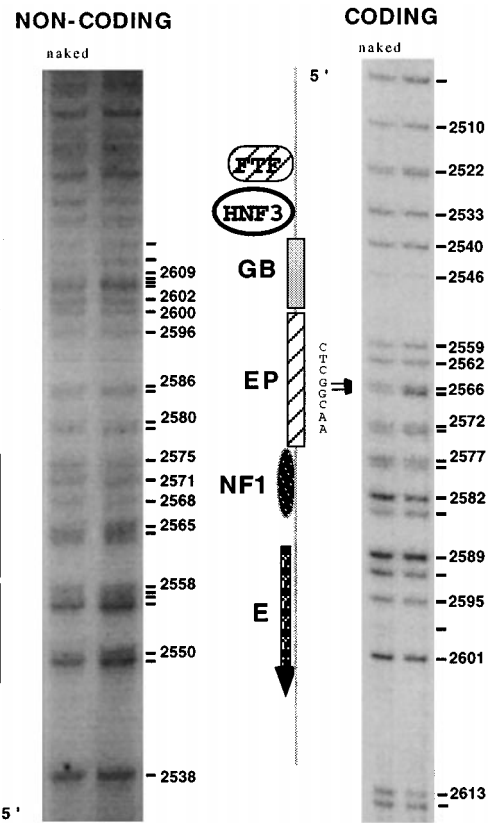


Figure 4 Genomic *in vivo* DMS footprinting of enhancer-I region of an HBV integrant, clone AI-26, in CHO cells. The analysis was performed as in Figure 3 but using CHO cells

footprinting to address this issue. Two different cell lines, PLC/PRF/5 (Alexander) and Hep3B, were analysed. We found that each harbors the enhancer-I region with extremely high sequence conservation. Furthermore, the enhancers are active *in vivo*. Finally, the pattern of protein interaction is similar to that previously detected in cell free systems. The finding that enhancer sequences are conserved and functional in these cell lines suggests that there is selection pressure for their long-term maintenance. Based on this consideration we propose that the HBV enhancer-I might play a role in hepatocellular carcinogenesis (see below).

DMS footprinting permits identification of the exact guanine residues that are in close contact with the binding proteins, and therefore is useful to determine the nature of the bound proteins as well. A major protected area corresponds to the previously characterized EP element. The protected G-residues are identical to that reported for the *in vitro* DMS footprinting of the EP (EF-C) element (Ostapchuk *et al.*, 1989), suggesting that the EP element in both cells bind at least the RFX1 protein (Blake *et al.*, 1996; Katan *et al.*, 1997; Siegrist *et al.*, 1993). In PLC/PRF/5 cells, the analysed EP element has an additional contact point with the binding protein at the non-coding strand due to a T to C transition.

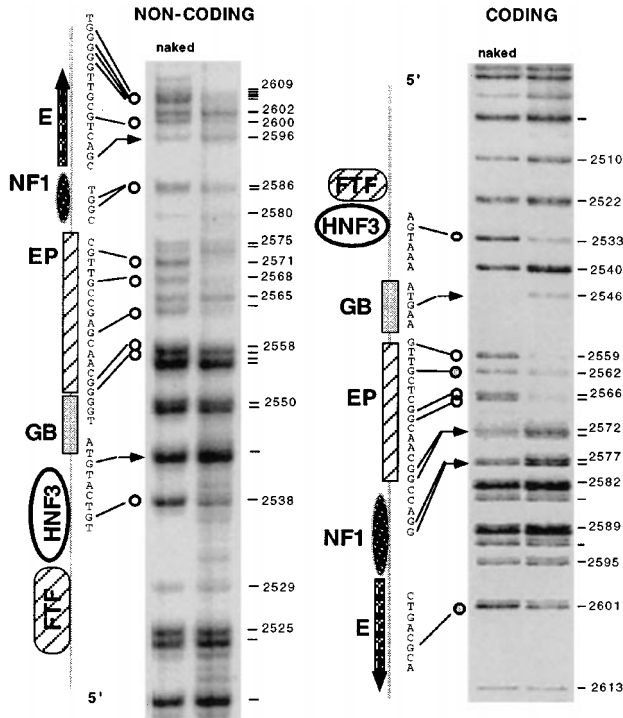


Figure 5 Genomic *in vivo* DMS footprinting of enhancer-I region of an HBV integrant in Hep3B cells. The analysis was performed as in Figure 3 but using Hep3B cells

In vitro analysis of the E element revealed that it binds multiple activators, mostly of the bZip family (C/EBP, AP1, CREB, ATFs). The genomic DMS footprinting of this element in PLC/PRF/5 cells shows a pattern of DMS protection similar to that of the AP1 occupation site (Konig, 1991). A different occupation pattern is seen in Hep3B cells (Figure 7). The fact that each cell line may utilize a different E-binding protein, is in agreement with our previous reports (Dikstein *et al.*, 1990b). At least some of the E-binding proteins are the very downstream targets of the cellular signaling pathway, and therefore, E element activity can be modulated by different external cues and environmental milieu (Faktor *et al.*, 1990). Interestingly, the E region *in vivo* shows strong DMS hyper-reactive G-residues in both tested cell lines, which were not detected *in vitro*. This is likely to result from association with other proteins in a manner that is not recapitulated *in vitro*.

In addition, our *in vivo* analysis revealed that FTF or a related protein binds enhancer-I. Previously, it was shown that a protein of about 50 Kd that was named PBF interacts with this cis element sequence (Kosovsky and Siddiqui, 1999). Given the fact that FTF binds and activates enhancer-II (Li *et al.*, 1998, Ishida *et al.*, 2000, Gilbert *et al.*, 2000), the role of this protein in HBV life cycle becomes significant. FTF is an orphan nuclear receptor of the *Drosophila* fushi tarazu F1 (FTZ-F1) family. FTF is a liver enriched transcription factor that plays a role in regulation of liver specific genes.

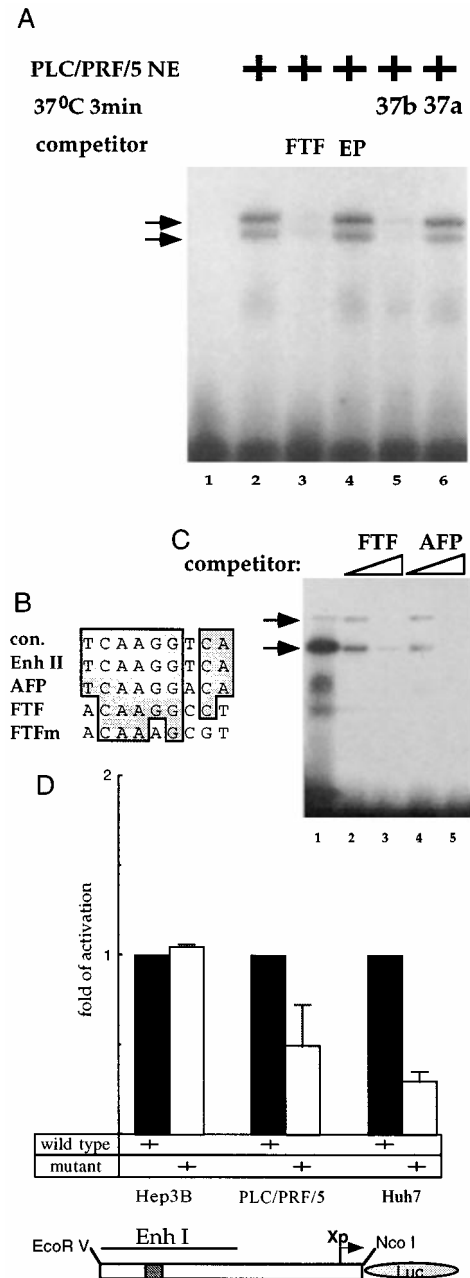


Figure 6 FTF binds and activates HBV enhancer-I. (a) Electromobility shifts with HBV FTF probe, and nuclear extracts from PLC/PRF/5 cells; lane 1 contains no extract, lanes marked FTF and EP contain 100-fold excess of cold oligonucleotides; in lanes marked 37b and 37a, the nuclear extracts were heated at 37°C for 3 min before and after incubation with the probe, respectively. (b) Sequence alignment of the fushi tarazu-F1 consensus sequence with those of the FTF binding site in HBV enhancer-II (Enh II), the α_1 -fetoprotein promoter (AFP), the HBV enhancer I (FTF), and the mutated binding site (FTFm). (c) EMSA as in (a) but with elevated competitor ratio, of 10 and 100-fold excess. (d) The activity of the enhancer was tested by transient transfection on a luciferase reporter gene under the control of HBV enhancer and pX promoter, containing either wild-type (black columns) or mutant FTF site (white columns). The results are normalized relative to the activity of the wild-type enhancer

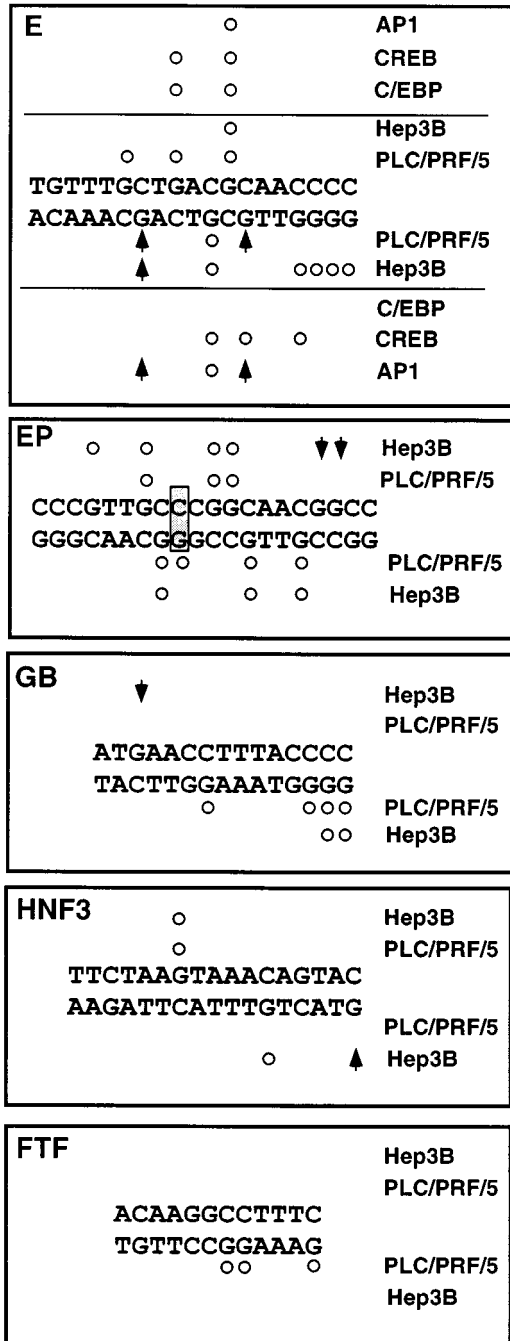


Figure 7 Schematic representation of the distinct HBV enhancer elements and the protected G residues. The sequences of the indicated elements with the protected G-residues (open circles) or hyper-reactive residues (triangles) at both strands and in the two analysed cell lines are shown. In the case of the E element, the protection pattern is compared with that reported for the indicated binding factors in a context of different genes (Bossard *et al.*, 1997; Datta *et al.*, 2000; Konig, 1991)

Both hepatocellular carcinoma cell lines analysed are active in production of HBsAg. In transfection experiments, the viral enhancer is important for supporting HBsAg production (Bulla and Siddiqui, 1988; Chang *et al.*, 1987; Faktor *et al.*, 1988). The fact

that both cell lines contain a functional enhancer lends further support to the role of the enhancer-I in driving HBsAg expression. It also has been reported that enhancer-II plays a role in regulating the synthesis of HBsAg (Zhou and Yen, 1990). Our analysis however suggests that this enhancer is inactive in the PLC/PRF/5 cells (data not shown). Thus, enhancer-II has only a minor role if any in regulating HBsAg expression.

Amplification of chromosomal band 11q13, where the HBV integrant Al-26 is mapped, is frequently observed in head and neck squamous carcinoma, oral squamous carcinoma, other digestive tract cancers, and breast carcinomas (Shuster *et al.*, 2000 and references therein). Additionally, significant cases of HCC were reported to be amplified at this chromosomal region (Kusano *et al.*, 1999; Sakakura *et al.*, 1999; Wong *et al.*, 2000), and a number of proto-oncogenes were mapped to this region. The fact that an HBV integrant with functional enhancer localized at this region might be important and may affect expression of a number of genes like the locus control regions (LCR). LCRs act at the level of chromatin remodeling and are necessary to ensure the establishment of an open chromatin configuration at highly heterochromatic regions, such as the centeromeres (Kioussis and Festenstein, 1997). Integration of HBV within the satellitic sequences characteristic of centeromeric regions was repeatedly reported (Miyaki *et al.*, 2000; Shaul *et al.*, 1986; Wang *et al.*, 2001; Chen *et al.*, 1994). At least in one case the integrant is transcriptionally functional (Miyaki *et al.*, 2000), strongly arguing that the integrated HBV DNA, like LCR, can lead to the opening of the heterochromatic configuration. According to this model, the HBV enhancer might have an intrinsic oncogenic function and therefore it might be considered as onco-DNA. The publication of the human genome sequence together with mapping of additional integrated HBV DNA in HCC samples will be instrumental for addressing this interesting possibility.

Materials and methods

Cell lines

PLC/PRF/5 (Alexander), and Hep3B cells were cultured in Dulbecco modified Eagle's minimal essential medium (GIBCO Laboratories) containing penicillin (100 U/ml) and streptomycin (100 mg/ml), supplemented with 8% fetal bovine serum.

In vitro DMS footprinting

For *in vitro* footprinting of HBV enhancers (either wt and mutants), the *Stu*I(1115)–*Sph*I(1235) DNA fragment was 5' ³²P end labeled (5–10 × 10⁶ c.p.m. per μg DNA) and incubated with nuclear extracts for binding reaction. The binding reactions were performed in 20 mM HEPES (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, 0.05 μM ZnCl₂, 2 mM dithiothreitol, 2 × 10⁴ c.p.m. of labeled DNA, 2 μg poly dI-dC and 40 μg of nuclear proteins (Ben-Levy *et al.*, 1989; Shaul and Ben-Levy, 1987). After incubation on ice for

30 min, 1 μ l DMS (Sigma) was added and the reaction incubated for 40 s. After phenol-chloroform extraction and ethanol precipitation, the samples were exposed to piperidine at 90°C for 30 min, ethanol precipitated and loaded on polyacrylamide urea sequencing gels.

In-vivo DMS footprinting

Monolayers of PLC/PRF/5, and Hep3B cells were cultured to subconfluence on 10 cm dishes. The cells were washed once with PBS at 37°C and treated with 0.5% DMS in PBS at 37°C for 2 min., then washed three times with PBS. DNA was extracted with TRI REAGENT (Molecular Research Center) according to the manufacturer's protocol. The extracted genomic DNA was cleaved at all methylated guanines by incubation in 1 M piperidine for 30 min at 90°C, followed by ethanol precipitation, drying the DNA pellet, and resuspension in 100 μ l of distilled water. Naked DNA from the same cells were subjected to DMS-piperidine cleavage and used as control.

DMS treated DNA was subjected to ligation-mediated PCR (LM-PCR) as described previously (HersHKovitz and Riggs, 1997). The oligonucleotides used for the analysis of the lower strand were: UP1: 5'-CCTTAATGCCTTTATATGC, UP2: 5'-ATATGCATGTATACAAGCAAAAACAGGC, and UP3: 5'-ACAAGCAAAAACAGGCTTTTACTTTCTCG. For the analysis of the upper-strand we used the following primers: DP1: 5'-GCAGTATGGATCGGC, DP2: 5'-CAAA-GGTTCCAGGCATGCGC, and DP3: 5'-TCCAGGCATGC-GCTGATGGCC. The asymmetric linker was identical to that previously described (HersHKovitz and Riggs, 1997).

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Electrophoretic mobility shift assays (EMSA)

Oligonucleotides used in EMSA were the following: HBV FTF: 5'-GATCCTTACAAGG CTTTCTA, AFP: 5'-CTTA-TGTTCAAGGACAAAGACC, and EP: 5'-GATCCGTT-GCT CGGCAACGGCCTA. Nuclear extracts were prepared as described (Dignam *et al.*, 1983). Binding reactions and electrophoresis were performed as previously described (Galarneau *et al.*, 1996), with 1 ng of ³²P labeled probe, 5 μ g PLC/PRF/5 nuclear proteins, and 10- or 100-fold molar excess of competitor oligonucleotides.

Luciferase assays

Cells were cultured and transfected as previously described (Haviv *et al.*, 1995). About 8 h before transfection, 2 \times 10⁵ cells were plated per 3.5 cm dish. Each dish was transfected with 30 ng of luciferase reporter plasmid containing HBV enhancer and either wild-type or mutant FTF sites (as described in Figure 6b, d), and 1 μ g of carrier DNA. 48 h post transfection, cells were harvested and assayed for luciferase activity.

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