Characterization of the hepatitis B virus major surface antigen promoter hepatocyte nuclear factor 3 binding site

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Transcription of the HBV 2·1 kb RNAs is regulated by the major surface antigen promoter. Previously, transient transfection analysis identified regulatory sequence elements in this promoter located between 189 and 1 which govern the level of transcription from this promoter and appear to bind only ubiquitous transcription factors including NF1, Sp1 and NF-Y. However, in vivo transcription analysis in transgenic mice has demonstrated that the expression of the HBV 2·1 kb RNAs is largely restricted to hepatocytes. In this study, the presence of a functional HNF3 transcription factor binding site located between 231 and 240 in the major surface antigen promoter suggests that the in vivo liver-restricted expression of the 2·1 kb RNAs may be governed by this liver-enriched transcription factor. The identification of a functional HNF3 binding site upstream of the DNA polymerase open reading frame also supports the contention that transient transfection analysis may fail to detect all of the cis-acting regulatory sequence elements involved in modulating the level of transcription from the viral promoters.

Introduction

The hepatitis B virus (HBV) genome is present in the nucleus of the infected hepatocyte as a 3·2 kb covalently closed circular DNA molecule (Ruiz-Opazo et al., 1982). This DNA is transcribed by the host RNA polymerase, synthesizing RNAs of 3·5, 2·4, 2·1 and 0·7 kb (Yen, 1993). In transgenic mice containing the complete HBV genome integrated into chromosomal DNA, the HBV transcripts are essentially restricted to the liver and kidney (Araki et al., 1988; Farza et al., 1988; Guidotti et al., 1995). Consistent with these observations, it is apparent that liver-enriched transcription factor is a major role in restricting HBV transcription to the livers of HBV transgenic mice. The enhancer II/nucleocapsid promoter, which controls the transcription of the 3·5 kb transcript, contains regulatory sequence elements binding the liver-enriched transcription factors C/EBP (Lopez-Cabrera et al., 1991; Yuh & Ting, 1991), RXRα (Raney et al., 1997), PPARα (Raney et al., 1997), HNF3 (Johnson et al., 1995) and HNF4 (Guo et al., 1993; Raney et al., 1997). The large surface antigen promoter, directing the expression of the 2·4 kb transcript, contains regulatory sequence elements which bind the liver-enriched transcription factors HNF1 (Zhou & Yen, 1991; Raney et al., 1991a) and HNF3 (Raney et al., 1995). The enhancer I/X-gene promoter region binds the liver-enriched transcription factors C/EBP (Dikstein et al., 1990; Pei & Shih, 1990), RXRα (Garcia et al., 1993; Huan et al., 1995), PPARα (Huan et al., 1995), HNF3 (Ori & Shaul, 1995; Kosovsky et al., 1996) and HNF4 (Garcia et al., 1993) and probably regulates the level of expression of the 0·7 kb transcript.

Whereas a role for liver-enriched transcription factors in the regulation of transcription of the 3·5, 2·4 and 0·7 kb transcripts has been indicated from transient transfection analysis in cell culture, similar analysis of the major surface antigen promoter, which governs the level of expression of the 2·1 kb HBV RNA, has not accounted for the liver-specific expression of this RNA in the HBV transgenic mouse model system. Transient transfection analysis of the major surface antigen promoter has indicated that the regulatory sequence elements governing transcription of the 2·1 kb transcript are located between −189 and the start site for transcription (Shaul et al., 1986; Raney et al., 1989, 1991b). Several transcription factors have been identified which bind within this region and regulate the level of transcription from the major surface antigen promoter. All of the identified transcription factors are ubiquitously expressed and include NF1 (Shaul et al., 1986), Sp1 (Raney et al., 1992) and NF-Y (Lu &
than direct activation of transcription. HNF3 may regulate viral RNA synthesis by mechanisms other than apparent in transient transfection analysis suggesting the liver-enriched transcription factor. In addition, the nature of this regulation is not evident in the polylinker of p19DLUC. pHBVTATA-LUC, CpB(3)TATALUC, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides were made by inserting one to seven copies (as indicated in the construct’s designation) of the HBV SpHNF3, SpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides into the unique Sall site or HindIII site of pHBVTATALUC. The oligonucleotide pairs used to generate the SpHNF3, SpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides were TCGATTAGGACACCCGG and TCGACCGTTGTTGCTGTA (oligo SpHNF3, HBV coordinates 2910 to 2931) (Fig. 1A), TCGACATATTTACACACTCTAGT and TCGACATAGTGTTAAATATGTG (oligo PpHNF1, HBV coordinates 2742 to 2763), TCGAAAGCTTGTGTTTAAAA and TCGA- TTAAACAAACAGCT (oligo CpE, HBV coordinates 1713 to 1729), TCGAAGTTATGTCACACTA and TCGAAGTTGTGTCGATACAA- CT (oligo PpHNF3, HBV coordinates 2161 to 2176) (Fig. 1B), TCGACTGGAGAGTT and TCGAAGCTCTCTCCACAG (oligo PpHNF, HBV coordinates 1730 to 1743), and AGCTGATTAATCATTCTC and AGCTGATTAATCATTCTC (oligo PpHNF1, HBV coordinates 2719 to 2734). The SpHNF3, PpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides span the major surface antigen promoter HNF3 binding site (Fig. 1A), the large surface antigen promoter HNF3 binding site (Raney et al., 1995), the nucleocapsid promoter HNF3 binding site 2 (Johnson et al., 1995), an HNF3 binding site upstream of the polymerase open reading frame (Fig. 1B), the nucleocapsid promoter Sp1 site 2 (Zhang et al., 1993), and the large surface antigen promoter HNF1 binding site (Raney et al., 1991a), respectively. The sequence of each construct was verified by dideoxy- nucleotide sequencing (Sanger et al., 1977).

The pCMVHNF3# vector expresses the HNF3# polypeptide from the HNF3# CDNA using the cytomegalovirus (CMV) immediate-early promoter (Pani et al., 1992). The pCMV vector, which lacks a cDNA insert, was generated by digestion of pCMVHNF3# with EcoRI and subsequent ligation. The pGEXHNF3# and pGEXHNF4# vectors express glutathione S-transferase (GST) fusion proteins in E. coli DH5α which include the complete HNF3# and HNF4# polypeptide sequence (Raney et al., 1995, 1997). Expression and subsequent affinity purification of the GST–HNF3# and GST–HNF4# fusion proteins using glutathione–agarose were performed as described by Smith & Johnson (1988).

**Cells and transfections.** The human hepatoma cell lines Huh7 (Nakabayashi et al., 1982) and HepG2.1 (Raney et al., 1990) were grown in RPMI 1640 medium and 10% foetal bovine serum at 37 °C in 5% CO₂/air. Transfections were performed as previously described (Graham & Van der Eb, 1973; Sorge et al., 1984) except that six-well plates, containing approximately 3 × 10⁵ cells per well, were used. The transfected DNA mixture comprised 5 µg of a LUC plasmid and 0.25 µg of pCMVβ, which served as an internal control for transfection efficiency. pCMVβ directs the expression of the E. coli β-galactosidase (β-gal) gene using the CMV immediate-early promoter (Clontech). The DNA mixture also included 0.5 µg of the HNF3# expression vector, pCMVHNF3#, or the control expression vector, pCMV, as indicated. Cell extracts were prepared 40 to 48 h after transfection. Cells were lysed in 150 µl of lysis buffer (0.1 M potassium phosphate, pH 7.8, 0.2%, v/v, Triton X-100) and the cell debris was pelleted by centrifugation for 2 min at 13,000 r.p.m. in a microcentrifuge. The supernatant was assayed for luciferase activity essentially as previously described (De Wet et al., 1987) and for β-galactosidase activity using a Galacto-Light kit (Tropix, Inc.) according to the manufacturer’s instructions.

**Whole-cell extracts, gel retardation analysis and DNase I footprinting.** Nuclear extracts were prepared from Huh7 as described previously (Dignam et al., 1983; Raney et al., 1989; Sladek et al., 1990). Gel retardation analysis was performed essentially as described pre-

Methods

**Plasmid constructions.** The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (Sambrook et al., 1989). The plasmids pHBVTATA-LUC, SpHNF3(5)TATALUC, SpHNF3(7)TATALUC, PpHNF3(3)TATALUC, PpHNF3(3)TATALUC, and PpHNF1(1)TATALUC were made by inserting one to seven copies (as indicated in the construct’s designation) of the HBV SpHNF3, SpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides into the unique Sall site or HindIII site of pHBVTATALUC. The oligonucleotide pairs used to generate the SpHNF3, SpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides were TCGATTAGGACACCCGG and TCGACCGTTGTTGCTGTA (oligo SpHNF3, HBV coordinates 2910 to 2931) (Fig. 1A), TCGACATATTTACACACTCTAGT and TCGACATAGTGTTAAATATGTG (oligo PpHNF1, HBV coordinates 2742 to 2763), TCGAAAGCTTGTGTTTAAAA and TCGA-TTAAACAAACAGCT (oligo CpE, HBV coordinates 1713 to 1729), TCGAAGTTATGTCACACTA and TCGAAGTTGTGTCGATACAA- CT (oligo PpHNF3, HBV coordinates 2161 to 2176) (Fig. 1B), TCGACTGGAGAGTT and TCGAAGCTCTCTCCACAG (oligo PpHNF, HBV coordinates 1730 to 1743), and AGCTGATTAATCATTCTC and AGCTGATTAATCATTCTC (oligo PpHNF1, HBV coordinates 2719 to 2734). The SpHNF3, PpHNF3, CpE, PpHNF3, and PpHNF1 double-stranded oligonucleotides span the major surface antigen promoter HNF3 binding site (Fig. 1A), the large surface antigen promoter HNF3 binding site (Raney et al., 1995), the nucleocapsid promoter HNF3 binding site 2 (Johnson et al., 1995), an HNF3 binding site upstream of the polymerase open reading frame (Fig. 1B), the nucleocapsid promoter Sp1 site 2 (Zhang et al., 1993), and the large surface antigen promoter HNF1 binding site (Raney et al., 1991a), respectively. The sequence of each construct was verified by dideoxy- nucleotide sequencing (Sanger et al., 1977).

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Fig. 1. (A) Nucleotide sequence of the HBV major surface antigen promoter region and (B) the nucleotide sequence upstream of the HBV DNA polymerase open reading frame (P ORF) (subtype ayw) (Galibert et al., 1979). The nucleotide coordinates are derived from the GenBank database and their position relative to a predominant transcription initiation site for the major surface antigen transcript (+1; nucleotide coordinate 3159) is shown in parentheses (Raney et al., 1989). The underlined sequences represent the HNF3 recognition sequence, the NF1 recognition sequence (Shaul et al., 1986), the Sp1 recognition sequences (Raney et al., 1992), and the NF-Y recognition sequence (Lu & Yen, 1996). The sequences of the HBV double-stranded oligonucleotides SpHNF3, SpA, SpB and PpHNF3 are indicated. The brackets under the sequences span the region protected from DNase I digestion by purified recombinant GST–HNF3α. The HBV major surface antigen promoter HNF3 recognition sequence and the HNF3 recognition sequence upstream of the polymerase open reading frame are shown, and the nucleotides homologous to the HNF3 consensus sequence (Roux et al., 1995; Kaufmann et al., 1995) are indicated (W = A/T, R = A/G; Y = C/T; M = A/C). The translational initiation codon (ATG) of the polymerase open reading frame is shown in bold.
Characterization of the DNA binding proteins in Huh7 nuclear extracts which interact with the HBV major surface antigen promoter and polymerase region HNF3 binding sites

DNase I footprinting and gel shift analysis has demonstrated that purified recombinant GST–HNF3α binds to the region between −231 to −240 of the major surface antigen promoter and upstream from the polymerase open reading frame between nucleotide coordinates 2164 and 2173. To examine further if HNF3 polypeptides specifically bind to these regions,
the PpHNF3 and SpHNF3 double-stranded oligonucleotides were characterized for their ability to bind proteins present in Huh7 nuclear extracts by gel shift analysis (Fig. 4). The PpHNF3 and SpHNF3 double-stranded oligonucleotides bound protein(s) present in the Huh7 nuclear extracts demonstrating that these sequence elements are recognized by transcription factor(s) present in this cell line. To examine the specificity of the complex formed between the PpHNF3 and SpHNF3 double-stranded oligonucleotides and the protein(s) present in the Huh7 nuclear extract, several unlabelled, double-stranded oligonucleotides were used as competitors in the gel retardation analysis (Fig. 4). Unlabelled, double-stranded oligonucleotides PpHNF3 (Fig. 1B), SpHNF3 (Fig. 1A), PS1pHNF3 (the HNF3 site in the large surface antigen promoter) and CpE (the HNF3 site 2 in the nucleocapsid promoter) efficiently competed with the $^{32}$P-labelled PpHNF3 and SpHNF3 double-stranded oligonucleotides for binding the factor(s) present in the Huh7 nuclear extract. The unlabelled major surface antigen promoter double-stranded oligonucleotides SpA (the NF1 site in the major surface antigen promoter)
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Fig. 4. Gel retardation and complex inhibition analysis of the HBV polymerase region PfHNF3 sequence element and the major surface antigen promoter SpHNF3 sequence element. The 32P-labelled, double-stranded oligonucleotides (A) PfHNF3 (Fig. 1B) and (B) SpHNF3 (Fig. 1A) were analysed using an Huh7 nuclear extract. Unlabelled, double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. PfHNF3 (lane 2), an HNF3 binding site located upstream of the polymerase open reading frame; SpHNF3 (lane 3), the major surface antigen promoter HNF3 binding site; PS1pHNF3 (lane 4), the large surface antigen promoter HNF3 binding site; CpE (lane 5), the nucleocapsid promoter HNF3 binding site 2 (Johnson et al., 1995); SpA (lane 6), the major surface antigen promoter NF1 binding site (Shaul et al., 1986); SpB (lane 7), the major surface antigen promoter Sp1 binding site 1 (Raney et al., 1992).

HNF3 can transactivate expression from a minimal promoter through the major surface antigen promoter and the polymerase region HNF3 binding sites

To examine the functional properties of the major surface antigen promoter and polymerase region HNF3 binding sites, they were examined for their capacity to mediate transcriptional transactivation by HNF3 in the context of a TATA-box element. The SpHNF3 and PfHNF3 double-stranded oligonucleotide synthetic promoter elements were cloned upstream of a TATA-box element and the luciferase open reading frame (LUC) and were tested for their transcriptional activities in HepG2.1 cells in the presence or absence of the HNF3β expression vector (Fig. 5). In the absence of the HNF3 expression vector, the constructs containing the major surface antigen promoter HNF3 site [construct SpHNF3(5)LUC and SpHNF3(7)LUC] had very little transcriptional activity. When the HNF3 polypeptide was exogenously expressed, the synthetic constructs containing the SpHNF3 sites were transcriptionally activated to a level similar to that observed for the HNF3 binding site in the HBV large surface antigen promoter [constructs PS1pHNF3(2)LUC and PS1pHNF3(3)LUC] (Raney et al., 1995), the nucleocapsid promoter [construct CpE(3)LUC] (Johnson et al., 1995) and upstream of the polymerase open reading frame [construct PfHNF3(3)LUC]. In contrast, transcription from the large surface antigen promoter HNF1 binding site [PS1pHNF1(1)LUC] and the nucleocapsid promoter Sp1 site 2 [construct CpB(3)LUC] was not activated by the exogenous expression of the HNF3 transcription factor. Therefore, the SpHNF3 double-stranded oligonucleotide containing the major surface antigen promoter HNF3 binding site appears to be a functional site which can
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Fig. 5. Functional analysis of the HBV major surface antigen promoter and polymerase region HNF3 binding sites. The constructs examined contain the SpHNF3 (the major surface antigen promoter HNF3 site), PS1pHNF3 (the large surface antigen promoter HNF3 site), CpE (the nucleocapsid promoter HNF3 site 2), PpHNF3 (the HNF3 site located upstream of the polymerase open reading frame), Cpb (the nucleocapsid promoter Sp1 site 2) and P1pHNF1 (the large surface antigen promoter HNF1 site) double-stranded oligonucleotide cloned into the minimal promoter construct pHBVTATALUC (see inset). The number of copies of the oligonucleotide in the construct is shown in parentheses in the construct name. The transcriptional activities of the constructs in the presence of the expression vectors, pCMV (-HNF3) or pCMVHNF3β (+HNF3), were examined in HepG2.1 cells. The transcriptional activities are reported relative to the SpHNF3(5)LUC plasmid in the presence of the HNF3β expression vector, which is designated as having a relative activity of 1.0. The standard deviation of the mean is indicated by an error bar. The internal control used to correct for transfection efficiencies was pCMVβ.

mediated specific transcriptional transactivation by HNF3β in the context of a TATA-box sequence to a level similar to that previously reported for the HNF3 binding sites in the large surface antigen and nucleocapsid promoters (Raney et al., 1995; Johnson et al., 1995). This observation is in apparent contrast to the observation that HNF3β only increases transcription from the major surface antigen promoter approximately 2-fold under conditions where transcription from the large surface antigen promoter is increased approximately 80-fold (Raney et al., 1995). Similarly, the PpHNF3 double-stranded oligonucleotide containing the polymerase region HNF3 binding site appears to be a functional site which can mediate specific transcriptional transactivation by HNF3β in the context of a TATA-box sequence to a level similar to that observed for the other HBV HNF3 binding sites. These results appear to demonstrate a limitation of the analysis of the viral promoters in cell culture by transient transfection as it failed to identify all the functional HNF3 binding sites in the HBV genome. A role for these additional HNF3 sites in determining the level of transcription from the HBV promoters in vivo may contribute to the differences in tissue specificity observed between viral transcription in transgenic mice or infected individuals and the patterns of transcription observed in cell culture.

Discussion

Studies aimed at understanding the mechanisms governing the coordinate and liver-specific regulation of HBV transcription have primarily been performed by transient transfection analysis using a variety of nonliver- and liver-derived cell lines. This approach has been successful in demonstrating the involvement of transcription factors such as NF1, Sp1, C/EBP, HNF3, HNF4, RXRα and PPARα in regulating the level of transcription from multiple HBV promoters which may explain, in part, both the coordinate regulation of HBV transcription and the preferential expression of HBV RNAs in liver-derived cell lines. However, these studies have failed to
explain why the tissue distribution of the 2-1 kb HBV transcript is primarily restricted to the liver and kidney of HBV transgenic mice (Araki et al., 1989; Farza et al., 1988; Guidotti et al., 1995), as only ubiquitous transcription factors had been identified in regulating the level of expression from the major surface antigen promoter (Shaul et al., 1986; Raney et al., 1992; Lu & Yen, 1996).

In this study, evidence is presented that a functional HNF3 binding site is located between −231 and −240 in the major surface antigen promoter. It is demonstrated by DNase I footprinting and gel shift analysis that purified recombinant GST–HNF3β polypeptide specifically binds to this region of the major surface antigen promoter. In addition, the major surface antigen promoter HNF3 binding site selectively binds factor(s) present in Huh7 nuclear extracts which results in complex(es) that can be inhibited from forming by known HNF3 binding sites including those present in the large surface antigen and nucleocapsid promoters. Functional analysis demonstrated that in the context of a minimal promoter the major surface antigen promoter HNF3 binding site was able to mediate HNF3-dependent activation of transcription in a manner similar to that previously observed for the HNF3 binding sites present in the large surface antigen and nucleocapsid promoters.

The apparent contradiction between the regulation of the expression of the 2-1 kb HBV transcript in cell culture studies and its highly tissue specific expression in HBV transgenic mice has potential implications for understanding the role of HNF3 in regulation of the synthesis of this transcript. Two relatively simple explanations may account for the experimental observations. First, it is possible that differences in the levels of active transcription factors in cell culture as compared with hepatocytes in vivo may affect the ability of HNF3 to activate transcription directly from the major surface antigen promoter. This assumes that the limited effect of HNF3 binding in cell culture reflects the efficient activation of transcription from the major surface antigen promoter by higher levels of active ubiquitous transcription factors present in cell culture as compared with hepatocytes in vivo. Consequently, binding of HNF3 to the major surface antigen promoter site in vivo, where ubiquitous transcription factors alone direct limited viral transcription, would dramatically increase transcription from the major surface antigen promoter, whereas in cell culture it would have little effect on the level of transcription from this promoter (Raney et al., 1995).

An alternative explanation for these observations relates to the nature of the transcriptional templates in the in vivo and cell culture analyses. In HBV transgenic mice, the HBV DNA template is integrated into the mouse chromosomal DNA and is presumably organized into nucleosomes. If HNF3 is essential for the disruption of the nucleosomal organization of the major surface antigen promoter and this step is required to permit the ubiquitous transcription factors to interact with the proximal promoter region, transcription of the viral 2-1 kb RNA might be restricted to hepatocytes where HNF3 is abundantly expressed (Lai et al., 1990, 1991). In contrast, in transient transfection analysis the major surface antigen promoter is present in transcriptional templates which probably have not been completely assembled into nucleosomes. In this situation, the ubiquitous transcription factors might have relatively free access to the major surface antigen promoter, permitting transcription of the 2-1 kb transcript to be relatively independent of the presence or absence of the HNF3 transcription factor, as is observed in transient transfection analysis (Raney et al., 1995). The observations that the HNF3γ transcription factor DNA binding domain has a structure similar to histone H5 (Clark et al., 1993) and the capacity of HNF3 to organize the localization of nucleosomes at the regulatory sequences of the mouse albumin enhancer (McPherson et al., 1993) support the idea that HNF3 might modulate transcription from the major surface antigen promoter by altering the chromatin structure of the viral minichromosome (Bock et al., 1994; Newbold et al., 1995). Additional experimental analysis will be required to determine if either of these possibilities can account for the difference in the observed cell-type specificity of transcription from the major surface antigen promoter observed in HBV transgenic mice in vivo and in transient transfection analysis in cell culture.

The inability to observe a functional role for the HNF3 binding site in the context of the major surface antigen promoter suggested that there may be additional HNF3 binding sites within the HBV genome which have gone undetected in standard transient transfection analysis. This appears to be the case as a functional HNF3 binding site was also observed upstream of the polymerase open reading frame (Figs 1 to 5). The significance of this observation remains unclear but suggests the possibility that either a weak promoter exists upstream of the polymerase open reading frame which may be responsible, in part, for the expression of this viral gene product, or that an unidentified enhancer element may exist in this region of the HBV genome. A cis-acting regulatory sequence element of this nature might function only under circumstances in vivo which are not observed in transient transfection analysis in cell culture, accounting for the previous failure to detect this regulatory element.

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