Characterization of the minimal elements of the hepatitis B virus large surface antigen promoter

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It has been demonstrated that the hepatocyte nuclear factor 1 (HNF1) binding site is critical for the majority of the hepatitis B virus (HBV) large surface antigen promoter activity in differentiated hepatoma cell lines. Examination of a series of clustered point mutations in the minimal large surface antigen promoter demonstrated that the HNF1 and TATA box binding sites are the major regulatory elements required for transcription from this promoter. Synthetic promoter constructs containing the large surface antigen promoter HNF1 binding site and TATA box element upstream of the luciferase open reading frame were tested for their transcriptional activities in HepG2.1 cells in the absence or presence of an HNF1 expression vector. These synthetic promoter constructs displayed a similar level of transcriptional activity and induction by HNF1 in comparison with the full-length large surface antigen promoter, suggesting that additional HBV sequences are dispensable for full transcriptional activity. The distance between the HNF1 binding site and TATA box element in the synthetic promoter constructs appeared to influence the transcriptional activity modestly and in a periodic manner.

Introduction

The mechanisms responsible for the highly restricted species and tissue tropism of hepatitis B virus (HBV) are currently under investigation. Replication of HBV may be restricted to hepatocytes for several reasons, including the requirement for liver-specific transcription factors for expression from the HBV promoters. It has been shown, in transgenic mice synthesizing HBV particles, that the major HBV RNAs are predominantly synthesized in liver and, to a lesser extent, in kidney tissue. This indicates that there is a tissue-specific transcriptional restriction in the expression of the HBV genes (Araki et al., 1989; Farza et al., 1988). Characterization of the cell-type specificity of the HBV enhancers and promoters has suggested that the enhancer elements and the core and large surface antigen promoters may direct preferential gene expression in hepatoma cell lines (Jameel & Siddiqui, 1986; Karpen et al., 1988; Antonucci & Rutter, 1989; Honigwachs et al., 1989; Yee, 1989; Raney et al., 1990; Chang et al., 1989; Patel et al., 1989; Shaul & Ben Levy, 1987; Yuh & Ting, 1993; Shaul et al., 1985; Tognoni et al., 1985), whereas the major surface antigen gene appears to be expressed efficiently in a range of tissue culture cell types (Pourcel et al., 1982; Siddiqui et al., 1986; De-Medina et al., 1988).

The large surface antigen promoter contains a sequence element that is necessary for maximal transcriptional activity which binds the highly liver-enriched transcription factor, hepatocyte nuclear factor 1 (HNF1) (Raney et al., 1990, 1991; Courtois et al., 1988; Lichtsteiner & Schibler, 1989; Frain et al., 1989; Chang et al., 1989; Nakao et al., 1989), and probably explains the preferential expression from this promoter in differentiated hepatoma cell lines. In the current study, the transcriptional activities of promoter constructs containing the minimal elements of the HBV large surface antigen promoter (Raney et al., 1990, 1991) were examined further in a transient transfection system. This analysis demonstrated that two elements, the HNF1 binding site and the TATA box, were sufficient to generate a level of transcriptional activity in the dedifferentiated hepatoma cell line HepG2.1 and the differentiated hepatoma cell line Huh7 similar to that of the complete large surface antigen promoter. The HNF1 induction of the synthetic promoter construct containing these two elements was also comparable to that of the large surface antigen promoter, indicating that other
HBV sequences were not necessary for maximal transcriptional activity. The distance between the two binding sites appeared to contribute to the level of transcriptional activity from these promoter constructs, suggesting the importance of spacing for the functional interaction of the HNF1 transcription factor with the basal transcription machinery.

**Methods**

**Plasmid constructs.** The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (Sambrook et al., 1989). The HBV DNA sequences in these constructs were derived from the plasmid pCP10, which contains two copies of the HBV genome (subtype ayw) cloned into the EcoRI site of pBR322 (Dubois et al., 1980). The firefly luciferase (LUC) reporter gene in these constructions was derived from the plasmid, p19DLUC (Raney et al., 1990). The plasmid PS1(p)LUC contains one complete HBV genome located directly 5' to the promoterless LUC reporter gene such that the expression of the LUC gene is governed by the hepatitis B virus large surface antigen promoter (Raney et al., 1990).

The plasmids M1LUC, M2LUC, M3LUC, M4LUC, M5LUC and M6LUC contain clustered point mutations that were generated by a two-step polymerase chain reaction (PCR) method using appropriate oligonucleotides to make the desired mutation within the HBV sequence (Landt et al., 1990). The PCR-generated mutant large surface antigen promoter fragments were subsequently cloned into the luciferase vector p19DLUC by standard procedures (Sambrook et al., 1989). The M1LUC, M2LUC, M3LUC, M4LUC, M5LUC and M6LUC plasmids are derivatives of the plasmid PS1(p)A2840-2707LUC and contain 10 nucleotide mutations in the region from the HNF1 binding site to the TATA box element in the large surface antigen promoter (Fig. 2a). The plasmid M5LUC is derived from the PS1(p)A2840-2425LUC construct and contains a 13 nucleotide mutation within the HNF1 binding site (Fig. 2a). The sequences of the fragments produced by PCR were verified by dideoxynucleotide sequencing (Sanger et al., 1977).

The plasmids pHNF1LUC, pHBV-TATALUC and pHNF1TATALUC were constructed by inserting synthetic double-stranded oligonucleotides into sites in the polylinker of p19DLUC. pHNF1LUC was made by inserting a double-stranded oligonucleotide containing the large surface antigen promoter HNF1 binding site, produced by annealing the oligonucleotides AGCTAGTTACAT TACTTC and AGCTGAAGTAATGATTAACT (spanning HBV coordinates 2719 to 2734), into the unique HindIII sites of p19DLUC in the same orientation as the TATA box element (Fig. 2a). The pHBV-TATALUC was constructed by inserting a double-stranded oligonucleotide containing the large surface antigen promoter TATA box element, produced by annealing the oligonucleotides CATTATATATAAGGAGAAGCT and TCTCCTTATATAATAGATTAC (spanning HBV coordinates 2773 to 2791), into the SacI and KpnI sites of p19DLUC in the same orientation as the TATA box element occurs in the HBV genome. pHNF1TATALUC was made by inserting the double-stranded oligonucleotide containing the large surface antigen promoter TATA box element between the SacI and KpnI sites of pHNF1LUC. A series of constructs was made in which the spacing between the HNF1 site and the TATA box element in the plasmid pHNF1TatalUC was changed by inserting various combinations of linkers between the two binding sites. The sequence of each construct was verified by dideoxynucleotide sequencing (Sanger et al., 1977).

**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 containing 10% fetal bovine serum at 37 °C in 5% CO<sub>2</sub>/air. The HepG2.1 cell line is a dedifferentiated hepatoma cell line which originated from the differentiated hepatoma cell line, HepG2, as described previously (Raney et al., 1990). The human cervical carcinoma cell line HeLa S3 (Puck et al., 1956) was grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose and 10% calf serum at 37 °C in 5% CO<sub>2</sub>/air. Transfections were performed as previously described (Graham & Van der Eb, 1973; Sorge et al., 1984). The transfected DNA mixture comprised 15 μg of a LUC plasmid and 1-5 μg of pSV2CAT, which served as an internal control for transfection efficiency. pSV2CAT directs the expression of the chloramphenicol acetyltransferase (CAT) gene using the SV40 early promoter (Gorman et al., 1982). When appropriate, the DNA mixture also included 1-5 μg of the HNF1 expression vector, pHNF1TatalUC (Raney et al., 1991), or the control expression vector, pMT. Cell extracts were prepared 40 to 48 h after transfection and assayed for luciferase and CAT activity essentially as previously described (Gorman et al., 1982; De Wet et al., 1987).

**Nuclear extracts and DNase I footprinting analysis.** Nuclear extracts were prepared from Huh7 cells, HepG2.1 cells and Hela S3 cells as described previously (Dignam et al., 1983; Raney et al., 1989). The DNase I footprinting assay was performed as described previously (Raney et al., 1989, 1992; Briggs et al., 1986). The reactions contained 1 to 5 ng of end-labelled DNA fragment in a 50 μl reaction mixture containing 25 mM-Tris-HCl pH 7.9, 6.25 mM-MgCl<sub>2</sub>, 0.5 mM-EDTA, 0.5 mM-DTT, 50 mM-KCl, 10% (v/v) glycerol and 4 footprinting units of purified TATA binding protein (TBP) (Promega), or 1 μg poly (dI-dC) and 50 μg of the nuclear extract to be assayed. Binding was carried out for 15 min at 0 °C and then for 2 min at room temperature, after which 50 μl of 5 mM-CaCl<sub>2</sub>, 10 mM-MgCl<sub>2</sub>, and DNase I, was added at room temperature. The reaction mixture was incubated for 2 min, after which the reaction was stopped by the addition of 100 μl of 1% (w/v) SDS, 20 mM-EDTA, 200 mM-NaCl containing 250 μg of tRNA per ml. The mixture was then phenol extracted, precipitated with ethanol and analysed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

**Results**

**Characterization of transcription factor binding sites in the HBV large surface antigen promoter**

The location of the TBP binding site in the large surface antigen promoter was determined by DNase I footprinting analysis with purified recombinant TBP (Fig. 1, lanes 1 and 2). The TBP footprint spanned the promoter region from −40 to −25 and included the A/T-rich sequence located between −36 and −25 which presumably represents the TATA box recognition element (Fig. 2a). DNase I footprinting analysis was also performed with the Huh7, HepG2.1 and HeLa S3 nuclear extracts to examine the role of differentiated-hepatoma-specific transcription factors in the regulation of transcription from the large surface antigen promoter (Fig. 1, lanes 3 to 6). Huh7 nuclear extracts protected the promoter region from −99 to −69 from DNase I digestion. This protected region included the 13 nucleotide sequence located between −89 and −77 which contains homology to the consensus recognition sequence for the transcription factor HNF1 (GTATAATNATTAAC) (Courtois et al., 1988). Con-
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1991), it seems likely that the protein binding this recognition site in the differentiated hepatoma cell line Huh7 is HNF1 or a related polypeptide. The absence of a detectable footprint over the HNF1 recognition sequence using the HepG2.1 and HeLa S3 nuclear extracts is also consistent with the observation that these cell lines lack transcription factors capable of trans-activating the large surface antigen promoter through the HNF1 recognition element.

Clustered point mutational analysis of the minimal large surface antigen promoter

A series of clustered point mutations (Fig. 2a) was introduced into the minimal large surface antigen promoter region between nucleotides −89 and −25 to determine whether any HBV sequences in addition to the binding sites for HNF1 and TBP play a role in the regulation of transcription from this promoter. The mutant large surface antigen promoter constructs were transfected into HepG2.1 cells in the presence of the negative control vector pMT, or the HNF1 expression vector pMTHNF1. In the absence of exogenously expressed HNF1, none of the clustered point mutations appeared to modulate the transcriptional activity from the large surface antigen promoter (Fig. 2b). In the presence of exogenously expressed HNF1, the mutant constructs M1LUC, M2LUC, M3LUC, M4LUC and M6LUC exhibited activities similar to those of the full-length promoter and their parental construct, PS(1)pA2840−2707LUC, and their levels of induction were also similar. These results indicate that these mutations do not appear to affect the activity of the large surface antigen promoter, in the absence or presence of exogenously expressed HNF1 in HepG2.1 cells.

In contrast, the M5 mutation in the large surface antigen promoter, which alters the HNF1 recognition sequence, did not influence the level of transcription from the promoter in the absence of HNF1 [compare PS(1)pA2840−2425LUC and M5LUC], but prevented the transcriptional trans-activation of this mutant promoter by exogenously expressed HNF1 (Fig. 2b). This observation reflects the absence of functional levels of HNF1 or HNF1-related polypeptides in HepG2.1 cells and the inability of the exogenously expressed HNF1 polypeptide to bind to the M5 mutant large surface antigen promoter.

The mutant large surface antigen promoter constructs were also transfected into Huh7 cells in the absence of the HNF1 expression vector to determine the effects of the mutations in the presence of endogenous HNF1. As predicted, the M5 mutation dramatically reduced the level of transcription from the large surface antigen promoter [compare PS(1)pA2840−2425LUC and
Fig. 2. Clustered point mutational analysis of the large surface antigen promoter. (a) The sequence (subtype ayw) of the HBV large surface antigen promoter region from -101 to +1 is shown. Coordinates of the large surface antigen promoter region are derived from the GenBank database and their position relative to the transcription initiation site (+1) is shown in parentheses. The regions of homology to the HNF1 recognition sequence and the TATA box element are indicated in bold typeface. The sequences protected from DNase I digestion by TBP and Huh7 nuclear extract are underlined. The sequence of the clustered point mutation is shown for each mutant construct. The nucleotides that differ from the HBV sequence are indicated in lowercase; unchanged nucleotides are indicated in uppercase in the clustered mutation and by dashes in the remaining minimal promoter sequence. The # in the M1 mutation indicates a one nucleotide deletion at this position. The t at coordinate 2753 in the M4 construct is a mutation introduced by PCR error. The M1, M2, M3, M4 and M6 mutations were introduced into the PS(1)pA2840-2707LUC construct and the M5 mutation was introduced into the PS(1)pA2840-2425LUC construct. (b) Relative activities of the clustered point mutation constructs in HepG2.1 cells in the
which is responsible for modulating the transcriptional efficiency in assembling the general transcription machinery at the large surface antigen promoter as a consequence of this mutation. The M1 and M2 mutations, which lie 3 to 12 nucleotides and 13 to 22 nucleotides, respectively, downstream of the HNF1 binding site each appeared to effect an approximately fivefold decrease in the transcriptional activity from the large surface antigen promoter in Huh7 cells. The reduced promoter activity due to the M1 and M2 mutations may indicate the presence of an additional regulatory transcription factor binding site in this region, which is responsible for modulating the transcriptional activity from the large surface antigen promoter. The factor binding this site would presumably be functional in Huh7 cells but absent or inactive in HepG2.1 cells.

Transcriptional activity from synthetic promoters comprising the large surface antigen promoter HNF1 binding site and TATA box element

It is apparent that the HNF1 binding site is a critical element necessary for the majority of the large surface antigen promoter activity in differentiated hepatoma cell lines [compare PS(1)pA2840–2425LUC and M5LUC, Fig. 2(b)]. In order to determine whether this promoter element alone, or in combination with the TATA box element could support transcriptional activity, synthetic promoter constructs containing these regulatory elements upstream of the luciferase open reading frame (ORF) were tested for their transcriptional activities in HepG2.1 cells in the absence or presence of the HNF1 expression vector (Fig. 3). The activities were reported relative to the activity of the full-length large surface antigen promoter construct [PS(1)pLUC] in the absence of exogenously expressed HNF1. The construct containing only the TATA box element upstream of the luciferase ORF (pHBVTATALUC) exhibited transcriptional activity approximately one-fifth of the level of the large surface antigen promoter and it was not inducible by exogenously expressed HNF1. The construct containing only the large surface antigen promoter HNF1 binding site upstream of the luciferase ORF (pHNF1LUC) produced transcriptional activity approximately two-fold lower than the full-length promoter and this activity was inducible to an extent similar to that of the large surface antigen promoter. The construct containing both the HNF1 binding site and the TATA box element (pHNF1TATALUC) displayed a level of transcriptional activity equivalent to the full-length large surface antigen promoter, suggesting that these two elements may be responsible for the majority of the activity from the large surface antigen promoter, and that the other HBV sequences are dispensable for full promoter activity. The transcriptional activity of this construct was also increased 10-fold by the expression of the HNF1 polypeptide, similar to the induction of the full-length promoter. These results suggest that transcriptional activity similar to that observed from the complete large surface antigen promoter can be achieved using a synthetic promoter comprising two promoter elements, the HNF1 recognition sequence and the TATA box element.

Influence of spacing between the HNF1 binding site and the TATA box element on the transcriptional activity from the large surface antigen promoter

In the synthetic construct pHNF1TATALUC, the spacing between the two promoter elements is 39 nucleotides, whereas in the large surface antigen promoter, there are 45 nucleotides between these two elements. The results shown in Fig. 3 suggest that this change in the spacing between the HNF1 binding site and the TATA box element may not greatly affect the activities or the inducibility of these constructs. However, to examine this issue, a series of plasmids was constructed from pHNF1TATALUC in which the spacing between the HNF1 binding site and the TATA box element was varied from 10 to 66 nucleotides. These constructs were transfected into HepG2.1 cells and their transcriptional activities in the absence and presence of exogenously expressed (+ HNF1) or absence (− HNF1) of exogenously expressed HNF1 polypeptide using the expression vectors pMTHNF1 and pMT, respectively, and in Huh7 cells in the absence of exogenously expressed HNF1 polypeptide are indicated. The activities are reported relative to the activity of the full-length promoter construct, PS(1)pLUC, in the absence of exogenously expressed HNF1 polypeptide. The internal control used to correct for transfection efficiencies was pSV2CAT. The diagrammatic representation of the construct PS(1)pLUC is shown. The HBV sequences are represented by the thin line. The locations of the HNF1 and TATA box sequence homologies are indicated by solid boxes labelled HNF1 and TATA, respectively. The luciferase gene (LUC) sequences are indicated by the open box. The locations of the M1, M2, M3, M4, M5 and M6 mutations are indicated by shaded boxes.
expressed HNF1 were compared with the transcriptional activity from the full-length large surface antigen promoter in the absence of exogenously expressed HNF1 (Fig. 4a). The results indicate that the spacing between the HNF1 recognition sequence and the TATA box element does not have a large effect on the activity from these synthetic promoters when examined in HepG2.1 cells in the absence of HNF1. This presumably reflects the absence of functionally important levels of transcription factors binding to the HNF1 site in HepG2.1 cells. In the presence of exogenously expressed HNF1 polypeptide, transcription was increased five- to 15-fold, with higher levels of relative activity being observed when the distance between the HNF1 and TATA binding sites was 18, 28, 38 or 50 nucleotides. One turn of the B form of the DNA helix spans approximately 10 nucleotides, thus the apparent 10 nucleotide periodicity of the higher transcriptional activities suggests that the relative angular orientation of the HNF1 transcription factor and TBP on the DNA helix influences the efficiency of transcriptional trans-activation from these synthetic promoter constructs. In Huh7 cells, which contain endogenous HNF1, relatively higher levels of activity were also observed when the distance between the HNF1 and TATA box recognition sequences was 18, 28, 38 or 50 nucleotides (Fig. 4b). This supports the idea that the relative positions of the HNF1 transcription factor and TBP on a promoter sequence can influence the level of transcription from the promoter to some extent. Superimposed on the periodic change in transcriptional activity, there appears to be a slight trend in both HepG2.1 and Huh7 cells of decreasing transcriptional activity as the distance between the binding sites is increased.

### Discussion

The HNF1 recognition sequence, GTTAATCATTACCT, located from -89 to -77 upstream of the start of transcription appears to be crucial for the majority of the activity from the large surface antigen promoter in differentiated hepatoma cell lines (Raney et al., 1990, 1991). This binding site is also necessary for the induction of the large surface antigen promoter by exogenously expressed HNF1 polypeptide in human hepatoma cell lines (Raney et al., 1990, 1991). This sequence is conserved between HBV genomes of the different viral subtypes (Raney & McLachlan, 1991), suggesting that it plays an important role in the life cycle of the virus. The liver cell type specificity of the large surface antigen promoter appears to be dependent upon the HNF1 transcription factor, which may contribute to the hepatotropism of the virus as the large surface antigen is an essential component of the envelope of the virus particle (Ueda et al., 1991; Bruss & Ganem, 1991). Consistent with this possibility, virus particles have been produced in transfection experiments only in highly differentiated hepatoma cell lines (Sureau et al., 1986; Tsurimoto et al., 1987; Sells et al., 1987; Chang et al., 1987; Yaginuma et al., 1987), which express the HNF1 polypeptide necessary to activate the large surface antigen promoter.
Fig. 4. The influence of spacing between the HNF1 binding site and the TATA box of the large surface antigen promoter in (a) HepG2.1 cells and (b) Huh7 cells. Synthetic promoter constructs containing the large surface antigen promoter HNF1 recognition sequence and the TATA box element separated by a range of nucleotide distances were transfected into (a) HepG2.1 cells in the absence (○) or presence (▲) of exogenously expressed HNF1 polypeptide, and into (b) Huh7 cells in the absence of exogenously expressed HNF1 polypeptide. The transcriptional activities are reported relative to the activity of the full-length PS(1)pLUC construct in the absence of exogenously expressed HNF1 polypeptide. The internal control used to correct for transfection efficiencies was pSV2CAT. The distance (nucleotides) indicates the number of nucleotides between the 3' and 5' boundaries of the HNF1 and TATA binding sequences, respectively. The constructs that generated peaks of relative activity are indicated by the distance between the binding sites (18, 28, 38 and 50 nucleotides).

Analysis of clustered point mutations in the minimal large surface antigen promoter indicates that the HNF1 and TBP binding sites are the major regulatory elements of this promoter. Transcriptional activity from the minimal promoter was unaffected by mutations introduced between the HNF1 and TBP recognition sequences in the absence or presence of exogenously expressed HNF1 in HepG2.1 cells, suggesting that sequences other than these two elements are not critical for the majority of the large surface antigen promoter activity in this system (Fig. 2). In Huh7 cells, the transcriptional activity of the minimal promoter was dependent upon the integrity of the HNF1 binding site, consistent with the observation that the endogenous HNF1 binding activity present in Huh7 cells footprints this promoter region (Fig. 1). Mutation of the sequences downstream of the HNF1 site, represented by the constructs M1LUC and M2LUC, also influenced the transcriptional activity of the minimal promoter in Huh7 cells. These mutations may have altered a recognition sequence for another transcription factor which is functional in Huh7 cells, but is either absent from or not functional in HepG2.1 cells. This clustered point mutation analysis suggests that although other regions of the minimal promoter appear to contribute to the maximal activity of the large surface antigen promoter, the HNF1 binding site is clearly the most critical element of this promoter. Without this element, the activity of the large surface antigen promoter is reduced approximately 20-fold in the Huh7 cell line, which expresses endogenous HNF1, and approximately 10-fold in HepG2.1 cells synthesizing recombinant HNF1 polypeptide (Fig. 2b).

Synthetic promoter constructs containing the HNF1 binding site with or without the TATA box element confirmed that these two elements were sufficient for maximal levels of HNF1-dependent transcriptional activity from the large surface antigen promoter (Fig. 3). These results show that additional HBV sequences are not necessary for HNF1-mediated transcriptional activation from this promoter. A previous report suggested that both the Oct-1 and HNF1 transcription factors were essential for HNF1-mediated transcriptional activation from the large surface antigen promoter (Zhou & Yen, 1991). However, the results of the clustered point mutation and synthetic promoter transfection analyses indicate that the Oct-1 binding site located between −61 and −54 is not necessary for HNF1-mediated transcriptional activation.

The level of transcription from the large surface antigen promoter appears to be modestly influenced by the distance between the HNF1 and TBP binding elements. The transcriptional activity is characterized by a 10 nucleotide periodicity, which probably reflects the importance of spatial organization for communication between the HNF1 transcription factor and the components of the general transcription machinery. A similar observation has been reported for the effect of spacing between the heat shock regulatory elements and the TATA-box sequence of a Drosophila hsp70 heat shock gene (Cohen & Meselson, 1988). The observation that the spacing between the large surface antigen promoter HNF1 recognition sequence and the TATA-box sequence is 45 nucleotides indicates that the two major regulatory elements are located in positions where their functional interaction is not favoured (Fig. 4). This suggests one reason why the large surface antigen promoter directs low levels of transcription relative to the major surface antigen promoter (Raney et al., 1990).
As overproduction of the large envelope polypeptide relative to the major envelope polypeptide (McLachlan et al., 1987; Ou & Rutter, 1987; Persing et al., 1986) would probably be detrimental to virus production, the observed organization of the large surface antigen promoter regulatory elements may serve to prevent inappropriate ratios of envelope polypeptides being synthesized. In this manner, transcriptional regulation is coupled to HBV biosynthesis.

This work was supported by Public Health Service Grant AI30070 from the National Institutes of Health and by the Stein Endowment Fund.

References


(Received 14 March 1994; Accepted 30 May 1994)