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Hepatocyte-Specific Expression of the Hepatitis B Virus Core Promoter Depends on Both Positive and Negative Regulation

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The core promoter of hepatitis B virus shows hepatocyte specificity, which is largely dependent on an upstream regulatory sequence that overlaps with viral enhancer II. Footprint analyses by numerous groups have shown binding by cellular proteins over a large stretch of DNA in this region, but the identity of these proteins and their role in core promoter function remain largely unknown. We present data showing that the transcription factor HNF-4 is one such factor, as it activates the core promoter approximately 20-fold via a binding site within the upstream regulatory sequence. Since HNF-4 is enriched in hepatocytes, its involvement at least partially explains the hepatocyte specificity of this promoter. In addition, however, we have found a region upstream of the HNF-4 site that suppresses activation by HNF-4 in HeLa cells but not in hepatoma cells. Therefore, the cell type specificity of the core promoter appears to result from a combination of activation by one or more factors specifically enriched in hepatocytes and repression by some other factor(s) present in nonhepatocytes, and it may provide a convenient model system for studying this type of tissue-specific transcriptional regulation in mammalian cells.

Hepatitis B virus (HBV) has a small circular DNA genome of about 3.2 kb that codes for at least seven primary translation products from four open reading frames (reviewed in reference 19). There are four promoters, which are activated by two separate enhancers. The core promoter (see Fig. 1) gives rise to transcripts coding for the viral polymerase and both forms of the core (capsid) protein, as well as the pregenomic RNA that is reverse transcribed to become the viral genomic DNA. Therefore, it is a key promoter for viral replication and morphogenesis. Numerous groups have shown that the core promoter shows strong hepatocyte specificity (6, 8, 16, 21), which may partially explain the hepatotropism of this virus.

The *cis* elements that are important for hepatocyte-restricted expression of the core promoter are just beginning to be defined, while the *trans*-acting factors that bind to these elements are largely unknown. Others have shown that the core promoter, like other promoters, can be divided into (i) a basal region that specifies the sites of transcriptional initiation and (ii) an upstream region that increases the efficiency of transcription in hepatoma cells (21). This upstream regulatory sequence (URS) of the core promoter coincides with the second viral enhancer (EnII) (18), which activates the surface gene promoter (22), and the laboratories of Siddiqui (9) and Ting (20) have shown that the transcription factor c/EBP and related factors bind to portions of EnII. Since these factors are hepatocyte enriched, this finding may partially explain the cell type specificity of the core promoter. However, c/EBP and related factors can act as negative regulators of transcription (7, 8, 12), and c/EBP is not expressed in hepatoma cells such as HepG2 (3), which nonetheless show high core promoter activity (21). Furthermore, Yuh and Ting (20) have shown that the URS, unlike EnII, does not function independently of position and orientation with regard to the core promoter. Therefore, distinct factors may be needed for this region of HBV DNA to function as the core URS versus its acting as EnII.

In view of the importance of this question for the HBV life cycle, we have investigated the *cis* elements and *trans*-acting factors needed for core promoter activity in hepatocytes. By cotransfection studies into HeLa cells, we show that HNF-4 is one hepatocyte-enriched transcription factor (15) that can activate the core promoter via its URS. Gel shift, footprint, and methylation interference analyses confirm the presence of an HNF-4 binding site in this region of the HBV genome. Surprisingly, a region upstream of this site can suppress the activation by HNF-4 in HeLa cells but not in HuH-7 hepatoma cells. Therefore, the hepatocyte specificity of the core promoter is due to a combination of up-regulation by at least one hepatocyte-enriched factor and repression by some other factor(s) that is present in nonhepatocytes.

MATERIALS AND METHODS

DNA plasmids. A promoterless plasmid, pUCAT, was first constructed by inserting the 1.6-bp *Hin*dIII-*Bam*HI DNA fragment of plasmid pBRCAT into the polylinker site of the pUC18 vector. This DNA fragment contains the coding sequence of the reporter chloramphenicol acetyltransferase (CAT) and the downstream polyadenylation sequence. An HBV DNA fragment (*Sph*I-*Fsp*I; map positions 1240 to 1806) (17) containing the core promoter and its URS was inserted in the unique *Hin*dIII site located upstream of the CAT coding sequence in pUCAT, thus creating plasmid pUCAT0. Plasmids pUCAT1, pUCAT7, pUCAT8, and pUCAT9 were constructed the same way except that the HBV DNA fragments used were *Bam*HI-*Fsp*I (nucleotides 1404 to 1806), *Sty*I-*Fsp*I (nucleotides 1646 to 1806), *Hin*cII-*Fsp*I (nucleotides 1688 to 1806), and *Stu*I-*Fsp*I (nucleotides 1705 to 1806), respectively.

Cell culture and DNA transfection. HeLa cervical carcinoma cells were grown in Dulbecco's modified essential medium containing 10% fetal bovine serum. HuH-7 hepa-

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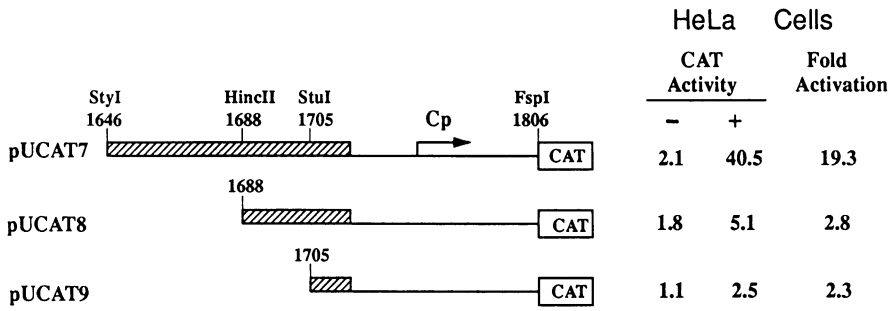

FIG. 1. Activation of the HBV core promoter by HNF-4. Construction of the DNA plasmids and the method for transfection of cells are described in detail in Materials and Methods. Cells were cotransfected with the reporter plasmid and the HNF-4 expression plasmid with or without HNF-4 coding sequence. The core promoter strength (CAT activity) was determined from the percentage of [^{14}C]chloramphenicol acetylated. Fold activation was calculated by dividing the CAT activity expressed in the presence of HNF-4 by that expressed in the absence of HNF-4. The results shown represent the averages of the results of at least three independent experiments. Cp, core promoter. Shaded boxes represent the EnII enhancer.

toma cells were grown in a medium containing a 1:1 ratio of Dulbecco's modified essential medium and F12 medium with 5% fetal bovine serum. The cells were transfected by the calcium phosphate coprecipitation method as previously described (5). Each 60-mm-diameter plate of cells was cotransfected with 2 μg of the reporter plasmid and 5 μg of the HNF-4-expressing plasmid pLEN4-S (15). For the control experiment, plasmid pLEN4-S was substituted with plasmid pLEN0. pLEN0 is the parental plasmid of pLEN4-S and does not contain the HNF-4 coding sequence (15). The CAT assay results were analyzed by thin-layer chromatography (5) and an Ambis image scanner. In most cases, 0.5 μg of plasmid pTKHGH (14) was included in each cotransfection experiment for monitoring transfection efficiency. pTKGH contains the human growth hormone sequence under the control of the herpesvirus thymidine kinase promoter (14). Transfection efficiency was measured by determining the amount of human growth hormone expressed from pTKGH. The amount of growth hormone expressed was measured by a commercial radioimmunoassay kit (Nicoles).

Nuclear extracts and DNase I footprint analysis. HuH-7 and HeLa nuclear extracts were prepared as described previously (5). For DNase I footprint analysis, the 650-bp *Eco*RI-*Sal*I DNA fragment of pUCAT6 containing the core promoter (nucleotides 1404 to 1806) and a part of the downstream CAT coding sequence was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as the probe. For DNase footprint analysis, 15 μg of the crude HuH-7 nuclear extract or 30 μg of the crude HeLa nuclear extract was preincubated with 2.5 μg of poly(dI-dC) on ice for 10 min in a 20-μl reaction mixture containing 12.5 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, and 5% glycerol. After addition of the DNA probe (approximately 0.05 pmol), the reaction mixture was further incubated at room temperature for 10 min. The DNase I digestion reaction was then carried out as previously described (4). For competition experiments, 50 ng of the double-stranded oligonucleotide competitor was added to the reaction mixture together with poly(dI-dC) prior to addition of the DNA probe. The sequence of the oligonucleotide competitor containing the HNF-4 binding site of the apolipoprotein CIII promoter (15) was

TCGAGCGCTGGGCAAAGGTCACCTGC
AGCTCGCGACCCGTTTCCAGTGGACG

The oligonucleotide containing the GCN4 transcription factor binding site was purchased from Stratagene.

In vitro translation and methylation interference experiments. To synthesize the HNF-4 RNA for in vitro translation, plasmid pSP64-HNF4 was constructed by inserting the *Bam*HI DNA fragment of plasmid pLEN4-S (15) into the unique *Bam*HI site of the plasmid vector pSP64 (Promega). This *Bam*HI fragment of pLEN4-S contained the HNF-4 coding sequence. pSP64-HNF4 was linearized with the restriction enzyme *Sph*I and used for RNA synthesis with SP6 RNA polymerase (Boehringer Mannheim) (11).

The protein translation reaction was carried out at 30°C for 1 h. A typical translation mixture contained 10 μl of rabbit reticulocyte lysate (Promega), 0.5 μl of 1 mM amino acid mixture, and 0.5 μg of HNF-4 RNA. For the gel shift assay, 2 μl of translation mixture was mixed with 2.5 μg of poly(dI-dC) and 11 μl of binding buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES; pH 7.9], 40 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM dithiothreitol, 4% Ficoll) and incubated on ice for 10 min. Approximately 100,000 cpm of the DNA probe was then added, and the reaction proceeded at room temperature for 20 min. The sample was then electrophoresed on a nondenaturing 5% polyacrylamide gel. The gel running buffer contained 25 mM Tris (pH 8.0), 25 mM sodium borate, and 0.25 mM EDTA. The DNA probe used for the gel shift assay was the *Sty*I-*Stu*I fragment (nucleotides 1646 to 1705) of HBV (17). For the competition assay, the oligonucleotide competitor was added into the reaction mixture prior to addition of the DNA probe. Methylation interference experiments were carried out by using previously described procedures (5).

RESULTS

Activation of core promoter by HNF-4. HeLa cells, which do not express HNF-4 and have previously been used for HNF-4 expression studies (15), were used to study the effect of HNF-4 on the HBV core promoter. To determine whether HNF-4 can activate the HBV core promoter, a CAT reporter plasmid driven by the core promoter with upstream sequences was cotransfected into HeLa cells together with an expression plasmid with or without the coding sequence for HNF-4. As seen in Fig. 1, the presence of HNF-4 resulted in an almost 20-fold increase of CAT expression. This increase was largely dependent on a small fragment of the upstream region, since a deletion of 44 bp (between the *Sty*I and *Hin*cII sites) essentially abolished the effect. Yuh et al. (21)

reported that deletion of this *Sty*I-*Hinc*II region could lead to reduction of more than 80% of the core promoter activity in both HepG2 and HuH-7 hepatoma cells. The most parsimonious interpretation of their results and ours is that the URS of the core promoter, between the *Sty*I and *Hinc*II sites, contains an HNF-4 binding site important for its function in hepatocytes.

Footprint analysis of core promoter. As the first step in confirming the presence of an HNF-4 site in the core URS, we used DNase protection analysis to localize sequences in the core promoter that bind nuclear factors present in HuH-7 but not HeLa cells. Since HNF-4 is not expressed in HeLa cells, the expectation was that by comparing the footprint patterns in these two cell types, a putative HNF-4 site can be identified. However, as seen in Fig. 2 and recently reported by Yuh et al. (21), numerous footprints were seen in both cell types upstream of the core transcript start sites, including the region between the *Sty*I and *Hinc*II sites. While closer inspection reveals fine differences in the footprint patterns in the two cell types (Fig. 2C), no sequence in this region that shows differential footprint patterns bears obvious homology to the consensus HNF-4 site.

As an alternative method to localize the putative HNF-4 site, we performed a competition footprint experiment. The DNase digestion was repeated in the presence of an excess of unlabeled competitor oligonucleotides containing either an authentic HNF-4 binding site from the apolipoprotein CIII promoter or a yeast GCN4 site. As seen in Fig. 3A, the latter oligonucleotide had no discernible effect on the footprint pattern obtained with HuH-7 extracts; in marked contrast, the HNF-4 oligonucleotide completely effaced the downstream portion of footprint 3. This was not a nonspecific effect, since no change in the HeLa footprinting pattern was seen with the same HNF-4 oligonucleotide (Fig. 3B). Therefore, the downstream portion of footprint 3 may contain a binding site for HNF-4.

Localization of the HNF-4 site. To confirm the footprint results and to localize more precisely the HNF-4 binding site, we performed gel shift and methylation interference assays. HNF-4 was synthesized by transcription and translation in vitro and used to bind a labeled fragment of HBV DNA containing this putative HNF-4 binding site (Fig. 2C). Specific binding was observed, since nondenaturing gel electrophoresis revealed a shifted band that was competed for by an unlabeled fragment bearing the authentic HNF-4 site but not by a fragment bearing the T7 promoter (Fig. 4).

The gel shift experiment was then repeated, but with DNA that had been lightly methylated with dimethyl sulfate. The DNAs within the bands corresponding to free DNA and HNF-4-bound DNA were recovered, cleaved at the methylated residues with piperidine, and electrophoresed on a sequencing gel. As seen in Fig. 5, the free DNA was methylated not only on all the guanine residues but also on many of the other residues to a lesser degree (evidently as a result of conditions during the methylation reaction that favored modification of non-G residues). However, in the case of HNF-4-bound DNA, methylation was not seen on a stretch of approximately 25 residues within the upstream portion of footprint 3 (Fig. 5). These residues define contact points between HNF-4 and HBV DNA in the region of footprint 3 (Fig. 6A), thereby confirming the presence of an HNF-4 binding site. This site shows partial identity to the consensus HNF-4 site (Fig. 6B) but is somewhat offset from the region defined by the competition footprint experiments shown in Fig. 3 (Fig. 6A). The discrepancy between the two sets of experiments can probably be explained by the presence of other factors that bind in this region (see Discussion).

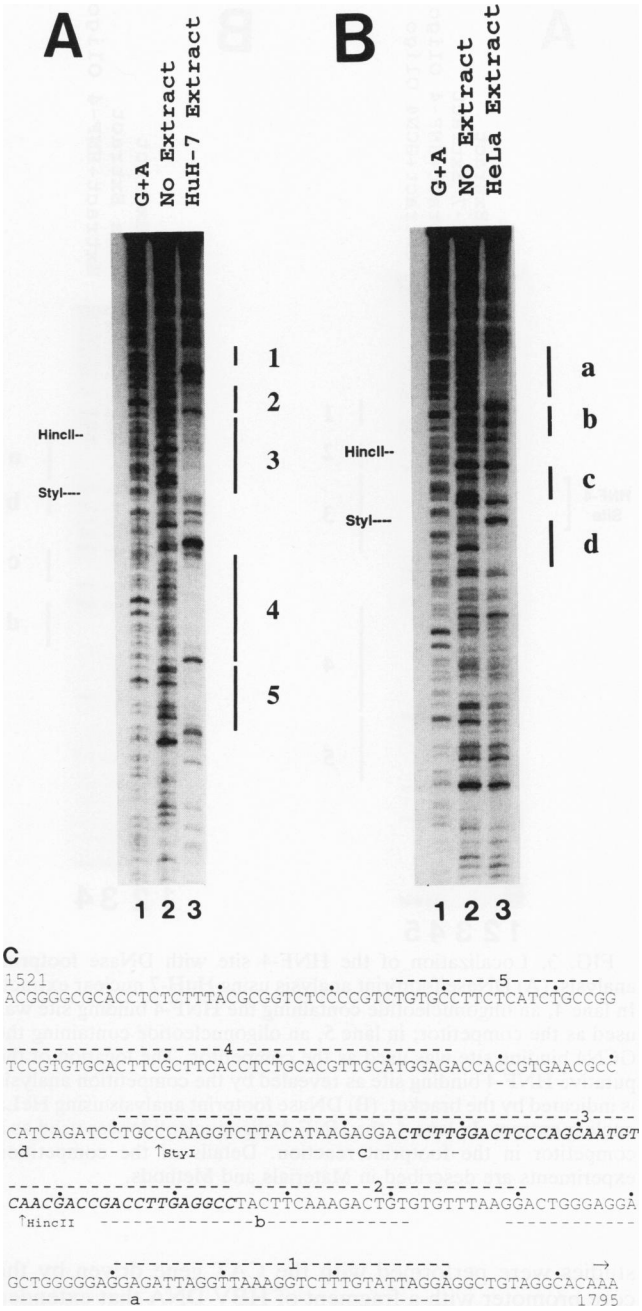

FIG. 2. DNase I footprint analysis of the core promoter and its URS. Details for the DNase footprint experiments are described in Materials and Methods. (A) DNase footprint analysis using HuH-7 nuclear extract. The five different footprints are indicated by numbers. (B) DNase footprint analysis using HeLa nuclear extract. The locations of the four footprints are indicated by a, b, c, and d. The *Hinc*II and *Sty*I restriction sites in the URS are also shown. (C) Locations of the footprints in the core promoter sequence. The putative HNF-4 binding site identified by the competition experiment is shown in boldface italics.

Presence of a dominant negative element in nonhepatocytes. In the course of these experiments, we noticed an apparently anomalous result. Specifically, when HNF-4 cotransfection

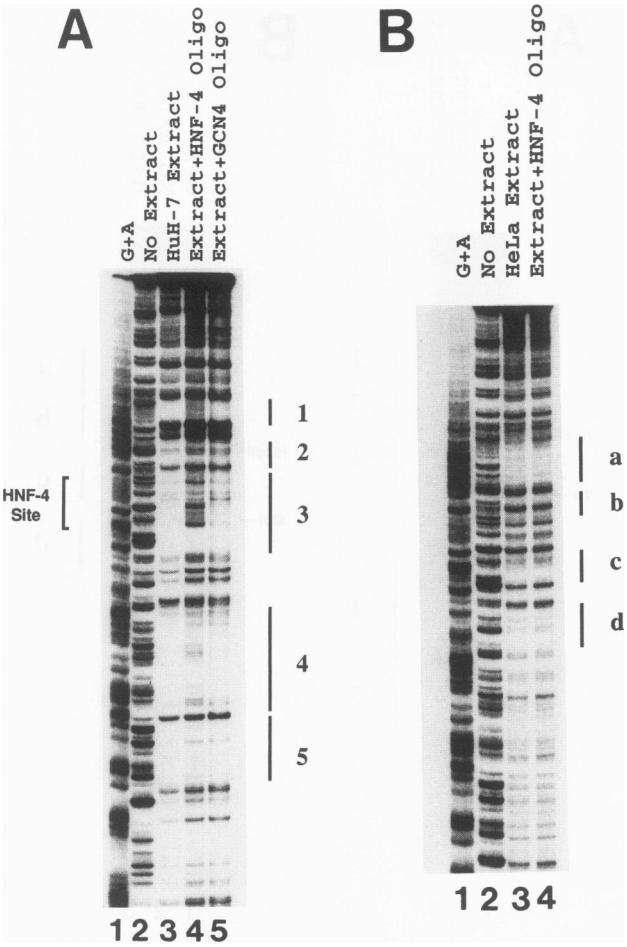

FIG. 3. Localization of the HNF-4 site with DNase footprint analysis. (A) DNase footprint analysis using HuH-7 nuclear extract. In lane 4, an oligonucleotide containing the HNF-4 binding site was used as the competitor; in lane 5, an oligonucleotide containing the GCN4 binding site was used as the competitor. The location of the putative HNF-4 binding site as revealed by the competition analysis is indicated by the bracket. (B) DNase footprint analysis using HeLa nuclear extract. In lane 4, the HNF-4 oligonucleotide was used as a competitor in the footprint reaction. Details of the competition experiments are described in Materials and Methods.

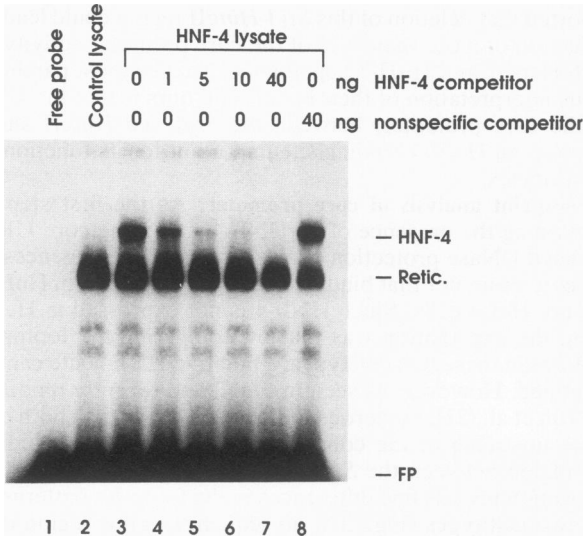

FIG. 4. Gel shift analysis for the binding of HNF-4 to the URS of the core promoter. Details of the gel shift assay are described in Materials and Methods. The tick mark labeled HNF-4 indicates the location of the band that could be abolished by the HNF-4 oligonucleotide competitor but not by the nonspecific oligonucleotide containing the T7 promoter sequence. The sequence of the nonspecific oligonucleotide competitor was

GTGAATTCTAATACGACTCACTATAGGGCG
CACTTAAGATTATGCTGAGTGATATCCCGCCTAG

FP, free probe; retic., a nonspecific band caused by the reticulocyte lysate.

studies were performed with the CAT gene driven by the core promoter with a fragment of HBV DNA that extended upstream of the HNF-4 site (pUCAT1 in Fig. 7), HNF-4 activation of the core promoter in HeLa cells was significantly suppressed. This result was reproducible. Furthermore, a similar result was obtained when a DNA construct including a even larger fragment of upstream sequences was used (pUCAT0 in Fig. 7). Therefore, there appears to be an upstream dominant negative element that can override activation by HNF-4 in HeLa cells. Similar experiments were done with HuH-7 hepatoma cells. Although HNF-4 activated CAT expression from the pUCAT7 DNA construct by almost 20-fold in HeLa cells, it has a much smaller effect in HuH-7 hepatoma cells, presumably because of the presence of endogenous HNF-4 in HuH-7 cells (unpublished observation). No apparent negative regulation of the core promoter by the upstream dominant negative element was observed in HuH-7 cells (Fig. 7), suggesting that the upstream sequences needed a cell-type-specific *trans*-acting factor for negative activity of the promoter. Therefore, in the context of the viral genome, the core promoter is inactive in nonhepatocytes not only because of the absence of HNF-4 but also because of the presence of a negative factor.

DISCUSSION

The HBV core promoter is an important regulatory element for the high-level expression of virion structural proteins and viral replication in infected hepatocytes. It has a high degree of cell type specificity (6), which is presumably important for the hepatotropism of HBV. In addition, because of its small size, the HBV genome can serve as a useful general model system for studying cell-type-specific transcription. Recently, the URS of the core promoter has been shown by Yuh et al. (21) to be critical for the high rate of transcription in hepatoma cells. In particular, they identified an important up-regulatory region (which they called CURS-A) that extends from map positions 1636 to 1703. However, the hepatocyte-enriched factors that activate the core URS had not been identified.

In this report, we have presented data showing that the URS of core promoter can be activated by HNF-4, a hepatocyte-enriched transcription factor. This activation is dependent on the presence of a URS fragment between the *Sty*I and *Hinc*II sites (map positions 1646 to 1688), which comprises the central portion of CURS-A. Nuclease protection analysis using hepatoma nuclear extracts revealed a footprinted region (map positions 1665 to 1705) that became sensitive to DNase digestion in the presence of an excess of oligonucleotides bearing an authentic HNF-4 site. Gel shift experiments with HNF-4 synthesized in vitro confirmed the

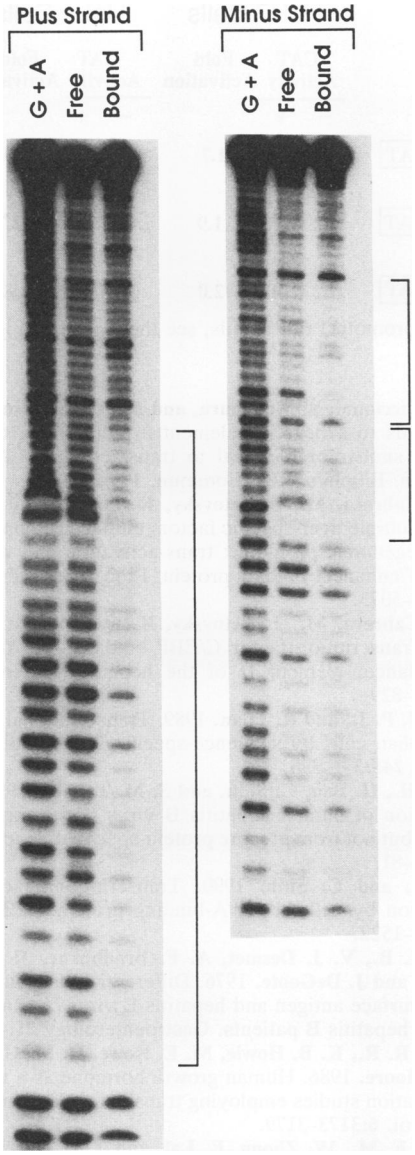

FIG. 5. Methylation interference analysis of the HNF-4 binding site in the core promoter. Details of the analysis are described in Materials and Methods. Brackets mark the locations of the footprints. Free, free DNA probe; Bound, the shifted HNF-4 band shown in Fig. 4.

presence of an HNF-4 site in this region, but methylation interference analysis mapped the binding site to positions 1650 to 1674, slightly upstream of the site defined by footprint competition. This apparent discrepancy can be partially explained by the fact that other factors seem to have binding sites that partially overlap with the HNF-4 site. For example, in HuH-7 cells, footprint 3 was partially effaced by the HNF-4 oligonucleotide competitor (Fig. 3A), suggesting the binding of HNF-4 and at least one other protein factor to this region. This separate factor(s) could mask a part of the sequence when HNF-4 binding is abolished. In addition, there may be other HuH-7 cell-specific factors that are recruited to this region of the DNA by HNF-4; this phenomenon would extend the apparent HNF-4 site in the competition footprint analysis. For these reasons, and because methylation interference is a more direct assay for the binding site, we believe that positions 1650 to 1674 contain the actual HNF-4 site. This conclusion is supported by the fact that the bottom strand in this region shows moderate homology (9 of 12 identical residues) to the consensus HNF-4 site (Fig. 6B). The core portion of this homologous region is actually repeated once in the opposite orientation immediately upstream (Fig. 6A). Therefore, it is possible that more than one molecule of HNF-4 binds in this region. If so, this would account for the relatively large size (25 bp) of the binding site identified by methylation interference.

Activation by HNF-4, a hepatocyte-enriched factor, can at least partially explain the hepatocyte specificity of the core promoter. However, the presence of HNF-4 appears to be insufficient to activate the core promoter in nonhepatocytes when sequences further upstream of the HNF-4 sites are present (i.e., in a situation similar to that found in the native viral genome). Deletional analysis reveals the presence of an upstream region that acts as a dominant negative element; i.e., it prevents HNF-4 function. This element presumably acts by binding to a cellular factor that either is not expressed or is inactive in HuH-7 hepatoma cells. Therefore, there are at least two layers of control to ensure hepatocyte-specific expression of the core promoter: activation by HNF-4, and probably other hepatocyte-enriched factors, and repression by a factor(s) present in nonhepatocytes.

The combination of positive and negative regulation in tissue-specific gene expression has been previously described for mammalian genes (for reviews, see references 2 and 10). For the HBV core promoter, the reason for this apparent duplication of effort may be ascribed to the fact that HNF-4 is not strictly liver specific; the kidney and intestine also express HNF-4 (15). Therefore, the presence of the negative element may be necessary to prevent inappropriate expression in nonhepatocytes that express HNF-4. Interestingly, transgenic mice bearing the entire HBV genome express the core gene in the liver and kidney but not the intestine (1). Therefore, it is possible that the negative *trans*-acting factor is present in intestinal cells but not kidney cells.

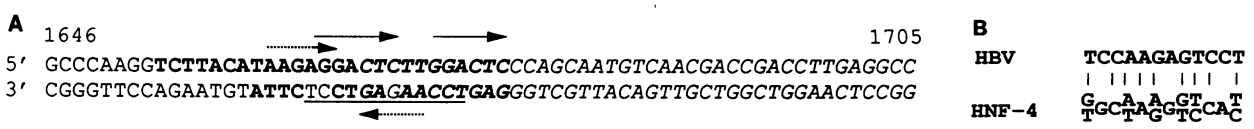

FIG. 6. (A) HNF-4 binding site in the core promoter as determined by methylation interference experiments. The nucleotides on which methylation interferes with the HNF4-binding are shown in boldface; the HNF-4 binding sequence as revealed by the DNase footprint experiment is shown in italics; the sequence homologous to the consensus HNF-4 recognition sequence is underlined; the inverted repeats of the core portion of the HNF-4 binding site are denoted by dashed-line arrows; two direct repeats which are also present in the HNF-4 binding sequence are denoted by solid-line arrows. (B) Comparison of the HNF-4 binding sequence in the HBV EnII enhancer (or URS of the core promoter) and the consensus HNF-4 binding sequence (15).

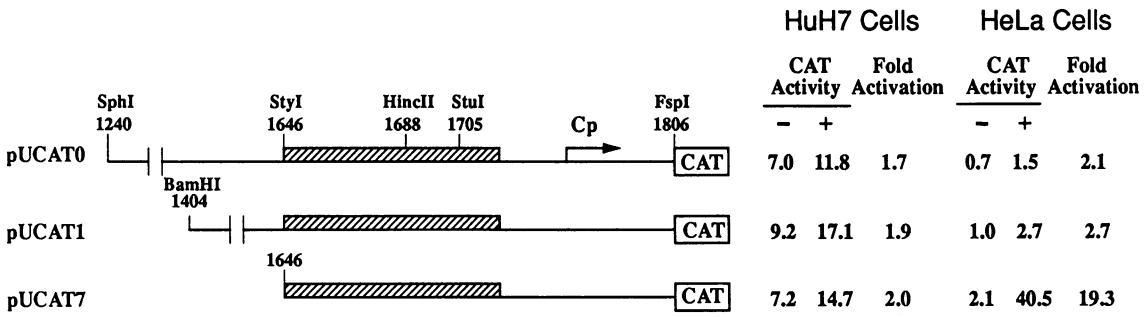

FIG. 7. Presence of a dominant negative element in the URS of the core promoter. For details, see the legend to Fig. 1.

Lastly, HNF-4 shows homology to the steroid/thyroid hormone family of receptors (15). The ligand for HNF-4, if any, has not been identified. Nevertheless, this finding raises the interesting possibility that an extracellular factor can potentially influence HBV gene expression. If so, this may explain why there is great variation in the level of HBV expression from person to person and even from hepatocyte to hepatocyte within the same person (13).

In summary, we have presented data showing that the HBV core promoter shows hepatocellular specificity for two reasons. First, the hepatocellular-enriched factor HNF-4 binds to the core URS and activates core gene transcription. Second, an as yet unidentified factor in nonhepatocytes represses core promoter activity via a site upstream of the core promoter. Because of the small size of the HBV genome, it will be relatively straightforward to identify the *cis*-element and *trans*-acting factor involved in this negative regulation. Therefore, the core gene may provide an ideal model system for studying tissue-specific transcription that depends on a combination of positive and negative regulation.

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