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# Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: Trans-activation and repression by CCAAT/enhancer binding protein

**(hepatitis B virus promoter mapping/CCAAT/enhancer binding protein binding/cotransfection with CCAAT/enhancer binding protein vector)**

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**ABSTRACT** **The human hepatitis B virus (HBV) is a hepatotropic virus that replicates through an RNA intermediate referred to as the pregenome. The promoter that directs the synthesis of the pregenome and several other transcripts with heterogeneous 5′ ends is of particular interest because of its role in regulating key functions during the viral life cycle. We have examined the liver-specific characteristics of this promoter by DNA–protein interactions and by demonstrating the *in vivo* function of the promoter using the luciferase reporter gene expression system. The DNA–protein interactions in this region appear to be almost entirely liver-specific. Among these, a liver-specific nuclear factor, CCAAT/enhancer binding protein, binds to at least five sites on this promoter. Transient cotransfection experiments using CCAAT/enhancer binding protein expression vectors and the core promoter in the context of either the native hepatitis B virus genome or the luciferase reporter gene demonstrate that CCAAT/enhancer binding protein at low concentration modestly activates expression from the core promoter but represses at high concentration.**

The human hepatitis B virus (HBV) causes acute and chronic hepatitis, and the infection has been associated with hepatocellular carcinoma (1). There are four genes encoded by the viral genome: *S/preS*, *C/e*, *Pol*, and *X* (1). The transcription of these genes is controlled by at least four promoters (1–7). Additionally, an enhancer element has been identified in the HBV genome (8, 9). The core or pregenomic promoter regulates synthesis of the 3.6-kilobase (kb) RNAs that include mRNAs for several viral proteins (core, e, and pol) and the pregenomic RNA. Transcriptional regulation of these viral macromolecules makes this promoter a key element of the viral life cycle. The core promoter exhibits enhancer-dependence for efficient liver-specific activity (6, 9–12).

In this study, we have used a series of restriction fragments and deletion mutations to define the boundaries of the HBV core/pregenomic promoter by using the firefly luciferase reporter gene (13). Furthermore, DNase I protection analysis was employed to identify nucleotide sequences that are binding sites for trans-acting cellular factors. This analysis revealed at least seven regions of DNA–protein interactions, most of which appear to be liver-specific. We show here that the CCAAT/enhancer binding protein (C/EBP), a liver-specific factor, binds to at least five sites in the promoter region. C/EBP was first purified from rat liver and shown to bind to several viral and cellular promoters/enhancers (14–18).

To assess the role of C/EBP in the core promoter function, cotransfection experiments were carried out with a C/EBP expression vector and the core promoter linked to a reporter gene or in the context of HBV genome. These studies indicate that C/EBP, at low concentrations, can function as a modest trans-activator of the core promoter. Increasing concentrations of C/EBP, however, resulted in trans-repression of the core promoter activity. Deletion mutations within the core promoter that destroy one of the C/EBP binding sites significantly affect overall promoter function.

## MATERIALS AND METHODS

**Plasmids and Transfection.** Defined HBV fragments (subtype adw), as indicated by the restriction sites (Fig. 1), were cloned into pXP2luc (20). Unless indicated as ΔE, every plasmid contained the HBV enhancer element [nucleotides (nt) 966–1308] upstream of the putative core promoter sequences. Deletion mutants (pAMSlucΔ12 and pAMSlucΔ71) were generated by cleaving with *Sty* I (nt 1645) and then treated with a combination of T4 DNA polymerase and S1 nuclease. Plasmids pMSV-C/EBP, pMSV-C/EBP-12V (21) and p18SYM were the generous gift of S. McKnight (Carnegie Institution of Washington). Plasmid pT109luc (20) was provided by S. Nordeen (University of Colorado Medical School). Transfected cell lysates were adjusted against β-galactosidase (pSVβ-galactosidase was used as an internal control) and then assayed for luciferase expression according to deWet *et al.* (13). Transfected cells with the recircularized enhancer-deleted HBV genome were assayed for HBV c/e antigen synthesis by a radioimmunoassay [ABBOTT HBe (rDNA); Abbott].

**DNase I Protection Analysis.** Nuclear extracts from rat liver and HeLa cells were prepared by the procedures described (15, 22). Recombinant (r) C/EBP synthesized in *Escherichia coli* was prepared by the method described by Landschulz *et al.* (17). DNase I protection analyses were performed as described (19). For footprint competition a CCAAT oligonucleotide (23) was used.

## RESULTS

**Functional Analysis of the Core/Pregenomic Promoter.** Restriction fragments and specific deletion mutations were generated from an *Ava* I–*Bgl* II fragment (nt 1466–1987) of the HBV genome that was identified as containing the core promoter activity (ref. 6; Fig. 1). These sequences were placed in front of the luciferase gene in plasmid pXP2luc (20) either alone or with the HBV enhancer. The promoter activities in these recombinant plasmids were assayed in two representative liver-derived cell lines: HepG2 (hepatoblastoma) and SK-Hep1 (nonhepatocytic adenocarcinoma). Lysates of transiently transfected cells were assayed for luciferase expression and the results are described in Fig. 1.

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Abbreviations: nt, nucleotide(s); C/EBP, CCAAT/enhancer binding protein; HBV, hepatitis B virus; r, recombinant; RLNE, rat liver nuclear extract; FP, footprint.

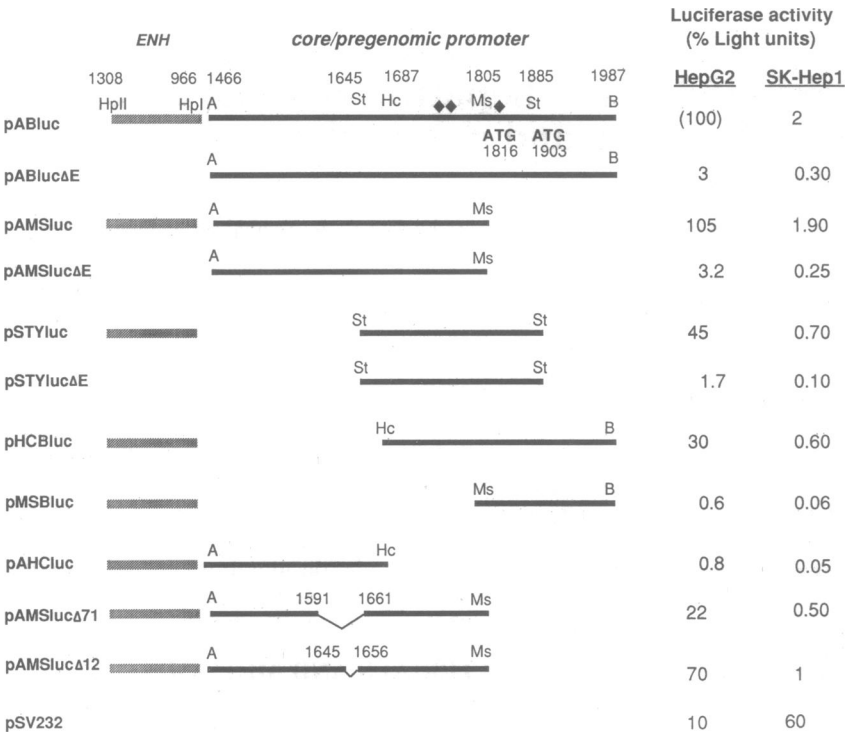


FIG. 1. Luciferase expression in HepG2 and SK-Hep1 cells. Schematic representation of HBV core promoter and enhancer (ENH) fragments inserted into the plasmid pXP2luc (19). The relative luciferase activity of transfected cells with each recombinant plasmid is indicated on the right. The luciferase activity of the plasmid pABluc in HepG2 has been set at 100%. The thin lines indicate the regions of internal deletions. Solid diamonds indicate the transcription initiation sites at nt 1745, 1751, and 1822. A, *Ava* I; B, *Bgl* II; Hc, *Hin*cII; HpI, *Hpa* I; HpII, *Hpa* II; Ms, *Mst* I; and St, *Sty* I. The plasmid pSV232 contains the luciferase gene under the control of the simian virus 40 early promoter (13).

Plasmids pABluc (*Ava* I–*Bgl* II, nt 1466–1987) and pAMSluc (*Ava* I–*Mst* I, nt 1466–1805) show the highest activities of luciferase and thus contain the sequence required for maximum activity of the core promoter in conjunction with the enhancer element. Their activities are 50- to 60-fold higher in HepG2 cells than in SK-Hep1. Removal of sequence in the 5′ end region of the core promoter upstream of the *Sty* I (nt 1645, pSTYluc) or *Hin*cII (nt 1687, pHCBluc) sites resulted in a 55% or 70% reduction of the activity, respectively. The sequence on the 3′ side of the *Mst* I site (nt 1805) did not show any significant promoter activity (pMSBluc) and deletion did not appear to alter promoter function (pAMSluc). A 12-base-pair (bp) deletion (nt 1645–1656) in the region upstream of the *Hin*cII site caused a 30% reduction of the promoter activity (pAMSlucΔ12) whereas an extended deletion of 71 bp (nt 1591–1661) reduced the promoter activity by 78% (pAMSlucΔ71). A similar level of reduction was also observed by deletion of the sequence upstream of the *Hin*cII site (pHCBluc). But a fragment containing this sequence alone (pAHCluc) was unable to produce any promoter activity. Thus these data suggest that the 232-bp sequence from nt 1591 to nt 1822 (the proximal transcription initiation site) contains all the necessary components for the core promoter activity. The initiation sites for all the viral transcripts and the pregenomic RNA are contained within this region of the core promoter (Fig. 1 and K.-Q.H. and A.S., unpublished data). The luciferase gene in the plasmid pSV232 (13) is under the transcriptional control of the simian virus 40 early promoter, which is expressed more efficiently in SK-Hep1 cells.

Removal of the enhancer (pABluc-ΔE, pSTYluc-ΔE, or pAMSluc-ΔE) reduces the promoter activity 30- to 40-fold in liver cells. This emphasizes the role of the HBV enhancer in stimulating transcription from the core promoter. The core promoter in the absence of the enhancer also displays preference for liver cells, being about 10-fold more efficient in HepG2 than in SK-Hep1 cells (Fig. 1). Similar results were obtained with other liver (Huh7) or nonliver (HeLa) cell lines (data not presented).

**Binding of C/EBP and Other Liver-Specific Factors.** An examination of the nucleotide sequence of the core promoter region revealed homology to the C/EBP binding sequence motif (14–16). DNase I protection analysis was performed to identify the binding sites of C/EBP and other factors to the core promoter. Two partially overlapping restriction fragments were used as radiolabeled probes: a distal fragment A (*Ava* I–*Hin*cII, nt 1466–1687) and a proximal fragment B (*Sty* I–*Sty* I, nt 1645–1885). The DNase I protection analysis of both coding and noncoding strands of fragment A revealed two distinct sites of binding with recombinant C/EBP (r-C/EBP), which are designated EBP1 and EBP1′ (Fig. 2*A*).

Crude rat-liver nuclear extracts (RLNEs) were also utilized in the DNase I protection analysis. Rat liver tissue was chosen as a convenient source of abundant amounts of liver-specific proteins. Also, this laboratory (11) has shown that the core promoter in conjunction with the HBV enhancer functions efficiently in rat hepatocytes. Three distinct footprints, designated FPI–III, were observed with fragment A in the presence of RLNE (Fig. 2*B*). An extension of FPIII was observed at the highest concentration of RLNE (150 μg of protein). Since C/EBP has been shown to be heat-stable factor (15, 23), we heat-treated RLNE prior to DNase I digestion to determine which of the footprints were produced by C/EBP. The pattern of the footprints shown in Fig. 2*B* demonstrates that heat treatment of RLNE abolished all the protected regions of fragment A except the sequence contained within FPIII that coincides with the site EBP1. This heat-stable footprint was competed with a CCAAT oligonucleotide (data not shown), which contains the C/EBP binding site. FPI, which coincides with the site EBP1′, does not appear with RLNE after heat treatment (Fig. 2*B*). A different heat-labile factor in RLNE may bind to this sequence. This was further substantiated by the failure of CCAAT oligonucleotide to compete for the binding seen at FPI (data not shown). Thus the EBP1′ footprint may have resulted from a relatively nonspecific interaction of r-C/EBP. HeLa extracts, which were used as a nonliver control, do not reveal any binding in this fragment (Fig. 2*B*). In summary, fragment A contains three liver-specific footprints (FPI–III), one of which, FPIII, includes a C/EBP binding site.

The DNase I protection analysis of fragment B is shown in Fig. 3. Footprints obtained with r-C/EBP are designated EBP1–5 (Fig. 3*A*). By using RLNE, five areas of DNA–protein

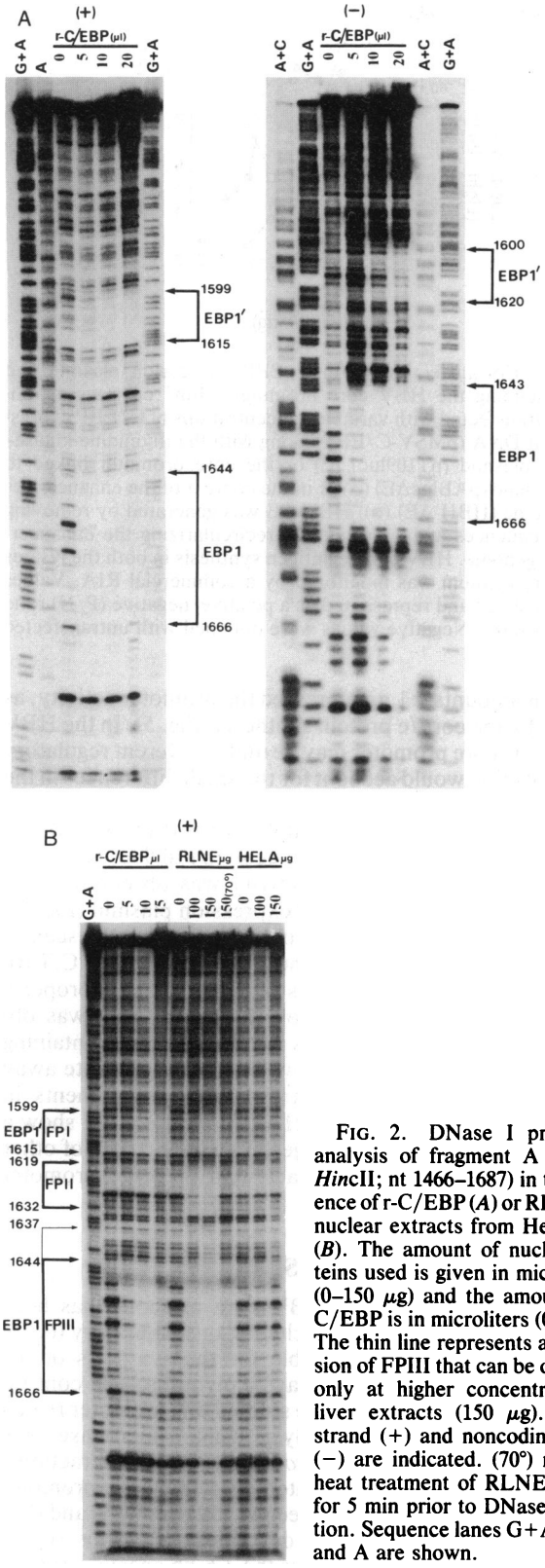


FIG. 2. DNase I protection analysis of fragment A (*Ava* I–*Hin*cII; nt 1466–1687) in the presence of r-C/EBP (*A*) or RLNE and nuclear extracts from HeLa cells (*B*). The amount of nuclear proteins used is given in micrograms (0–150 μg) and the amount of r-C/EBP is in microliters (0–20 μl). The thin line represents an extension of FPIII that can be observed only at higher concentration of liver extracts (150 μg). Coding strand (+) and noncoding strand (−) are indicated. (70°) refers to heat treatment of RLNE at 70°C for 5 min prior to DNase I digestion. Sequence lanes G+A, A+C, and A are shown.

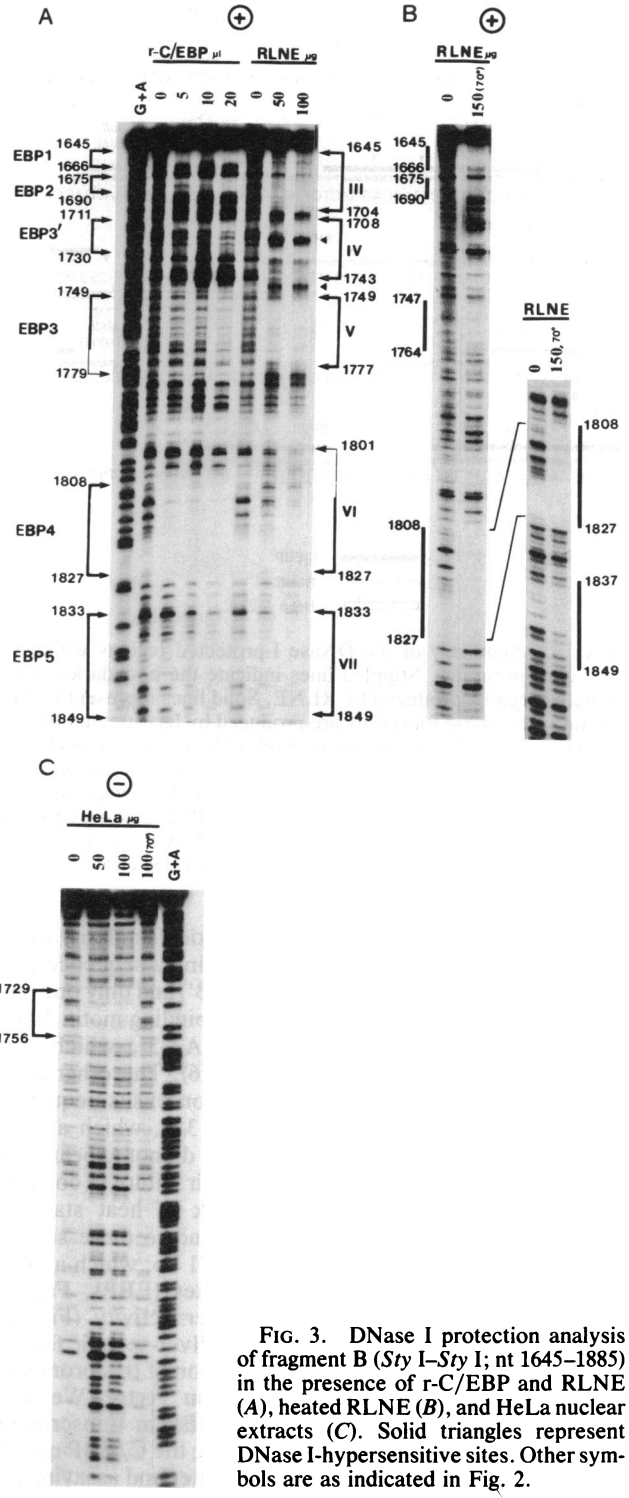


FIG. 3. DNase I protection analysis of fragment B (*Sty* I–*Sty* I; nt 1645–1885) in the presence of r-C/EBP and RLNE (*A*), heated RLNE (*B*), and HeLa nuclear extracts (*C*). Solid triangles represent DNase I-hypersensitive sites. Other symbols are as indicated in Fig. 2.

interaction (FPIII–VII) were observed within the coding strand of this fragment. It is interesting to note that a 20-bp sequence (nt 1667–1686) in FPIII that is protected using fragment B does not seem to be protected with fragment A. This may suggest the importance of surrounding sequences and interactions between multiple factors in producing the complete protection of the sequence in question. After heat treatment of the RLNE, five footprints were seen that coincide with sites EBP1, -2, -4, and -5 and partially with site EBP3 produced by r-C/EBP (Fig. 3*B*). Binding to these heat-stable areas of protection was competed with the CCAAT oligonucleotide and thus represents binding sites for C/EBP in liver cell extracts (data not shown). A different heat-labile factor in RLNE appears to occupy EBP3′ site. It is also apparent that the extended FPIII seen with RLNE is created by at least two factors binding, one of which is C/EBP. By using HeLa nuclear extract, a single heat-labile footprint was obtained overlapping partially with FPIV and FPV (Fig. 3*C*).

In summary, there are seven areas of DNA–protein interaction in the core promoter, the majority of which appear to be liver-specific (Fig. 4). Furthermore, a liver-enriched nuclear factor, C/EBP binds to at least five distinct sites. Of

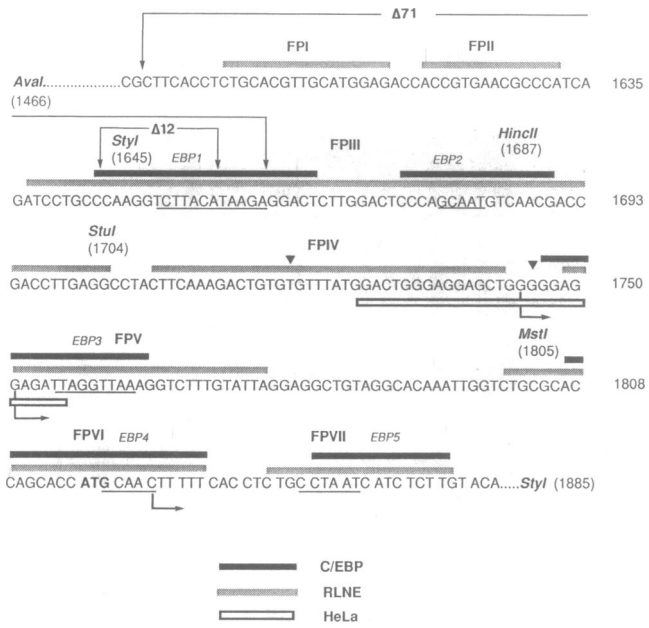


FIG. 4. Summary of the DNase I-protected regions within the HBV core promoter. Stippled lines indicate the boundaries of the protected regions produced by RLNE. Solid lines represent C/EBP binding sites. These footprints are produced by heat-treated RLNE, are competed by CCAAT oligonucleotide (23), and also coincide with r-C/EBP binding sites. The open box indicates the boundaries of the protected region by HeLa nuclear extracts. Solid triangles indicate DNase I-hypersensitive sites. Sequences with homologies to consensus C/EBP binding motifs are underlined. Arrows indicate the cap sites of the three species of the 3.6-kb RNA. Deletions of 12 and 71 bp are also indicated.

these, sites EBP1 and EBP2 exhibit striking similarity to the consensus sequence motif. EBP1 contains a sequence with dyad symmetry (5′-TCTTACATAAGA-3′) and only one mismatch relative to the canonical C/EBP binding motif. EBP2 contains the pentanucleotide 5′-GCAAT-3′, which is a known binding sequence for C/EBP (16). The other three binding sites show variations of the consensus sequence. Sites EBP1′ and EBP3′ (Figs. 2*A* and 3*A*), which are observed only in the presence of r-C/EBP, do not appear to be occupied by C/EBP in RLNE due to their failure to compete with CCAAT oligonucleotide and lack of heat stability. Apparently, other heat-labile factors bind to these sites in liver cell extracts. Deletions of 12 and 71 bp, which abolish one of the stronger C/EBP binding sites (EBP1, Fig. 4), significantly affected the core promoter activity (Fig. 1). Thus, C/EBP in conjunction with other liver-specific factors may be crucial in transcriptional regulation of this promoter.

**Cotransfection with C/EBP Expression Vector.** We have investigated the functional role of C/EBP in transcription from the core promoter by cointroducing the C/EBP encoding molecules along with the core promoter and assaying the response. Transient cotransfections of liver cells (Huh7 and HepG2) with pABlucΔE (Fig. 1) and increasing amounts of the C/EBP expression vector pMSV-C/EBP (21) were performed (Fig. 5). A 4-fold stimulation of core promoter-linked luciferase expression was observed with 50–200 ng of C/EBP expression plasmid. However, at higher concentrations the core promoter was repressed. The maximum repression was 10- to 15-fold in the presence of 10–20 μg of C/EBP DNA during cotransfection.

To assess the effect of C/EBP in the context of the native core/e gene expression, the recircularized enhancer-deleted HBV genome was cotransfected with the C/EBP expression vector. In this case, an ≈2-fold stimulation of the core promoter activity was observed with 0.1–1 μg of C/EBP

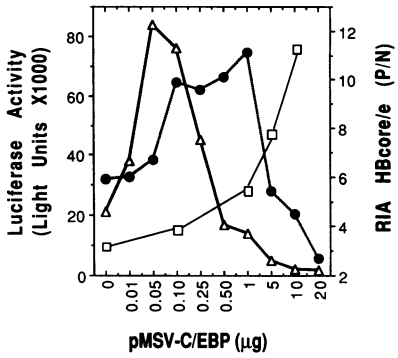


FIG. 5. Cotransfections with C/EBP expression plasmid and DNA containing the HBV core promoter. Huh7 cells were transiently cotransfected with variable concentrations of C/EBP expression vector DNA (pMSV-C/EBP) along with the thymidine kinase–luciferase plasmid (pT109luc) (□) or the core promoter linked to luciferase gene (pABlucΔE) (△) or in the context of the enhancerless HBV genome (HBH-ΔE) (●). HBV-ΔE was generated by removing plasmid sequences and subsequently recircularizing the enhancerless HBV genome. HBV core/e antigen synthesis in both the lysates and culture medium was monitored by a commercial RIA. Values were normalized and represented as a positive/negative (P/N) ratio as described (6). Negative values were obtained with untransfected cells.

DNA, but amounts >1 μg repressed the promoter activity, as detected by the core/e protein synthesis (Fig. 5). In the HBV genome, the core promoter may be under different regulatory constraints that would account for the small differences in the levels of induction and repression seen with these two systems. By using the herpes simplex virus thymidine kinase promoter linked to the luciferase gene (pT109luc, ref. 20), a 10-fold increase of luciferase activity was observed in the presence of 10–20 μg of C/EBP expression plasmid (Fig. 5). No increase or decrease of luciferase activity was seen in cotransfections with a vector encoding the mutated C/EBP pMSV-C/EBP-12V, which lacks the DNA-binding property (data not shown). Similarly, no effect of C/EBP was observed in triple cotransfections with a plasmid containing CCAAT multimers (p18SYM), which should compete away C/EBP (data not shown). Cotransfection experiments in nonhepatocytic SK-Hep1 or HeLa cell lines did not show a response to C/EBP, which suggests the importance of other liver-specific factors that interact with the core promoter (data not shown).

## DISCUSSION

The liver specificity of the HBV core promoter has been described by several groups, including this laboratory (6, 10, 12). The promoter is responsible for the synthesis of the 3.6-kb RNAs and, therefore, plays a pivotal role in controlling the virus life cycle. We have studied this promoter region by both *in vivo* functional analysis using a luciferase gene expression system and by *in vitro* DNA–protein interactions. These studies have demonstrated that the core promoter includes at least the 260 bp between nt 1591 and 1850 and thus is larger than that previously described (10). This region includes all the initiation sites for the 3.6-kb species of RNA and contains all the DNase I footprints observed with nuclear extracts from liver cells. The data presented here and those reported by Yaginuma and Koike (24) suggest that the nucleotide sequence downstream of the *Hin*cII site (nt 1687) is essential for promoter activity. Our results further indicate that the sequence upstream of the *Hin*cII site (up to nt 1591) is also important for the core promoter activity. This is supported by the presence of several liver-specific DNA–protein interactions in this region and a higher level of

luciferase expression upon inclusion of those sequences in transfection experiments. In a recent report, a cis-acting repressive element was described in the sequence upstream of the *Hin*cII site (25), but our data did not reveal such an effect.

In the present analysis of the core promoter, we provide evidence that multiple liver-specific proteins, including C/EBP, bind to specific sequences (Fig. 4). The identity of the other liver factors is presently unknown. Identification of C/EBP-specific footprints in the presence of crude liver extracts was established by the appearance of heat-stable footprints that were also competed by its cognate sequence, the CCAAT oligonucleotide. The binding of C/EBP to the core promoter in the presence of crude liver extracts may result from interactions of C/EBP with other transcriptional factors in a combinatorial manner. However, when partially purified r-C/EBP is used, such a situation does not exist. In this case, additional C/EBP footprints (EBP1′ and EBP3′, Figs. 2*A* and 3*A*) were observed that may have resulted from binding to cryptic sites, facilitated by the presence of a greater amount of C/EBP than is found in RLNE. The two liver-specific DNA–protein interactions that have been reported (24, 26) are included in FPIV and FPIII.

In cotransfection experiments, a C/EBP expression vector induced a 2- to 4-fold stimulation of the core promoter activity but only at low concentration, whereas at higher concentrations of C/EBP DNA the promoter activity was repressed (Fig. 5). Under similar conditions, the herpes simplex virus thymidine kinase promoter (pT109luc, ref. 20) exhibited a maximum of 10-fold stimulation by C/EBP (Fig. 5). Friedman *et al.* (21) have shown similar trans-activation of the albumin promoter in cotransfection experiments of HepG2 cells with the C/EBP expression vector. The trans-repression of the core promoter activity may be the result of C/EBP binding to sites that are normally not bound by this protein. In this case, C/EBP binding may preclude the interactions of other liver-specific transcriptional factors that play a positive role in the overall activity of the promoter. Another possible explanation for the observed repression may be the "squelching" of transcription factor(s) by C/EBP. However, this possibility seems less likely in light of the cotransfection experiment, in which both thymidine kinase–luciferase and core promoter–chloramphenicol acetyltransferase vectors were transfected together with increasing concentrations of C/EBP DNA. Under these conditions, the thymidine kinase promoter activity was stimulated and the core promoter activity was repressed (data not presented).

Binding sites for C/EBP have been identified on a number of viral and liver-specific gene promoters/enhancers (14–18, 20, 23). For HBV, C/EBP interacts with the core promoter in at least five major areas and three other sites in the HBV enhancer (ref. 23; J.L. and A.S., unpublished data). We have also observed a similar repression of the HBV enhancer activity in cotransfections with the C/EBP encoding plasmid vector (unpublished results). Whether or not the apparent repression of the core promoter or the enhancer activity has any physiological significance remains to be examined. In view of these studies, it is conceivable that variable levels of C/EBP in infected hepatocytes might be effective in controlling the HBV gene expression. C/EBP has been shown to be limited in tissue distribution and is especially enriched in liver (27). In addition, the level of cellular C/EBP has been linked to differentiation states of liver cells (21, 27). For example, the endogenous level of C/EBP expressed by HepG2, a hepatoblastoma cell line, is an order of magnitude lower than that found in differentiated hepatocytes (21).

Yee (25) has reported the presence of a liver-specific enhancer activity in the sequence described here as the core promoter. In our observations, the sequence from nt 1687 to nt 1805 also displays enhancer activity in the context of simian virus 40 early promoter–luciferase (unpublished results). How such an activity might influence the HBV gene expression and in particular the core promoter function remains to be understood. The present analysis of DNA–protein interaction may thus represent functional domains of this second putative enhancer. In light of these studies, the hepatotropism of the HBV, besides being controlled at the receptor level, may be directly influenced by the liver-specific transcriptional regulation of the core promoter.

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