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Functional analysis of a liver-specific enhancer of the hepatitis B virus

(liver-specific transcription/hepatitis B liver factor)

MIGUEL A. TRUJILLO, JOHN LETOVSKY, HUGH F. MAGUIRE, MANUEL LOPEZ-CABRERA, AND ALEEM SIDDIQUI

Department of Microbiology and Immunology and Program in Molecular Biology, University of Colorado Medical School, Denver, CO 80262

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ABSTRACT **The liver-specific enhancer I of the human hepatitis B virus contains several regions of DNA–protein interaction. Located within this element are also the domains of a promoter controlling the synthesis of the X open reading frame. Functional domains of the enhancer I and the X gene promoter were identified using DNase I protection analysis, deletion mutagenesis, and cell transfections. A unique liver-specific interaction was identified within this element whose binding site includes a direct sequence repeat, 5′-AGTAAA-CAGTA-3′. The factor(s) binding to this sequence motif was purified by oligonucleotide-affinity chromatography. Binding of this factor appears to play a key role in determining the overall enhancer function. Additionally, the interaction of several purified factors is presented. Cotransfection of liver cells with expression vectors encoding transcriptional factors resulted in trans-activation of the promoter/enhancer function. Based on the results of genetic analysis a model outlining the functional domains of the enhancer/promoter region is presented.**

The human hepatitis B virus (HBV) infects hepatocytes and causes acute and chronic hepatitis. Expression of the HBV genes appears to be modulated by transcriptional control elements that display liver specificity. This is facilitated by the interaction of trans-acting cellular factors with upstream regulatory sequences. There are four genes encoded by the viral genome (Fig. 1*A*): *S/preS*, *C/e*, *Pol*, and *X* (1), whose transcription is controlled by four promoters (see review, ref. 1). Two enhancer elements have been identified in the HBV genome by the use of reporter genes in heterologous systems (3–5), both of which display liver specificity (3–7). The enhancer element, termed enhancer I, is located between 966 and 1308 nt (3, 4, 7) and also has been speculated to contain the functional domains of the X gene promoter. This enhancer has been previously shown to direct its influence on the promoters of C, X, and, to a modest extent, the S ORF (8–12). Deletion of this enhancer from the genome results in a general decline of transcription from viral promoters (13). Analysis of DNA–protein interactions of both enhancers has revealed binding regions for several cellular transcriptional factors (6, 14–17). These protein binding sites display homology to known sequence motifs for which factors have been identified and purified. Some of these interactions have been shown to be liver specific (6, 14–17). A second element, designated as enhancer II, was identified relatively recently (5) and maps approximately within the core/pregenomic promoter at nt 1645–1803 in the HBV adw2 genome.

In this report, using deletion mutagenesis studies of the enhancer/X promoter complex, we have identified key elements crucial for enhancer function and those required for X promoter activity. Two regions of DNA–protein interaction were shown to be essential for enhancer activity. The transcription factor EF-C binds to one of these sites (18), and the other site binds a unique liver-specific factor that we have purified. Regions of DNA–protein interaction essential for the X promoter activity are also defined.

Using a transient cotransfection scheme of expression with vectors encoding transcriptional factors, we demonstrate the possible functional relevance of these interactions.

MATERIALS AND METHODS

Cells and Transfections. Transient transfections were performed with human liver-derived cell lines, HepG2 (hepatoblastoma) and Huh-7 (hepatocarcinoma). The plasmid pSV2β-galactosidase was used as an internal control to normalize transfection values. Luciferase assays were performed according to deWet *et al.* (19).

Plasmids. Luciferase expression plasmids either contained the simian virus 40 (SV40) early promoter (pSluc2) or lacked a promoter (pXPIluc or pXP2luc) (20). pSV-C/EBP was constructed by cloning the 1.1-kilobase *Nco* I fragment of the C/EBP ORF (21) under the control of the SV40 promoter/enhancer. Plasmid pMSV-C/EBP contains the C/EBP ORF under the control of the murine sarcoma virus long terminal repeat (22). pRSV-cJun was a gift of Michael Karin (23).

Protein Preparations and DNase I Protection Assay. Nuclear extracts (RLNEs) were prepared from fresh rat livers as described by Johnson *et al.* (2). Recombinant C/EBP (r-C/EBP) was prepared by the method of Landschulz *et al.* (21) from the *Escherichia coli* strain BL21 (DE3) pLysS harboring the plasmid pT5-42K (gift of S. McKnight). NF-1 and AP-1 (gifts of W. Dynan) were purified from HeLa cells using the methods of Rosenfeld and Kelly (24) and Kadonaga and Tjian (25), respectively. The latter method was also used to purify protein(s) binding to FPV. The DNase I protection analyses were performed as described (26). The reaction products were subjected to electrophoresis on 8% polyacrylamide gels containing 8 M urea.

Polymerase Chain Reaction (PCR)-Mediated Mutagenesis. HBV enhancer sequences (nt 966–1375) were cloned into the *Hinc*II site of plasmid pGEM3 (Promega). PCR was performed with selected oligonucleotide primers (27). The annealing temperature was set at 37°C. Amplified sequences were blunt-ended with T4 DNA polymerase and cloned into the *Hinc*II site in pGEM3. The extent of the deletions produced and the orientation were verified by dideoxy sequencing (Amersham). The luciferase gene derived from pSluc2 (20) was inserted downstream of the PCR-generated enhancer sequences between the *Bam*HI and *Eco*RI sites. For defining the X promoter functional domains, sequences from *Hpa* I (966 nt) to *Nco* I (1372 nt) and the PCR-derived enhancer deletion mutations were cloned in front of a pro-

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Abbreviations: nt, nucleotide(s); ORF, open reading frame; FP, footprint; RLNE, rat liver nuclear extract; SV40, simian virus 40; HBV, hepatitis B virus; r, recombinant.

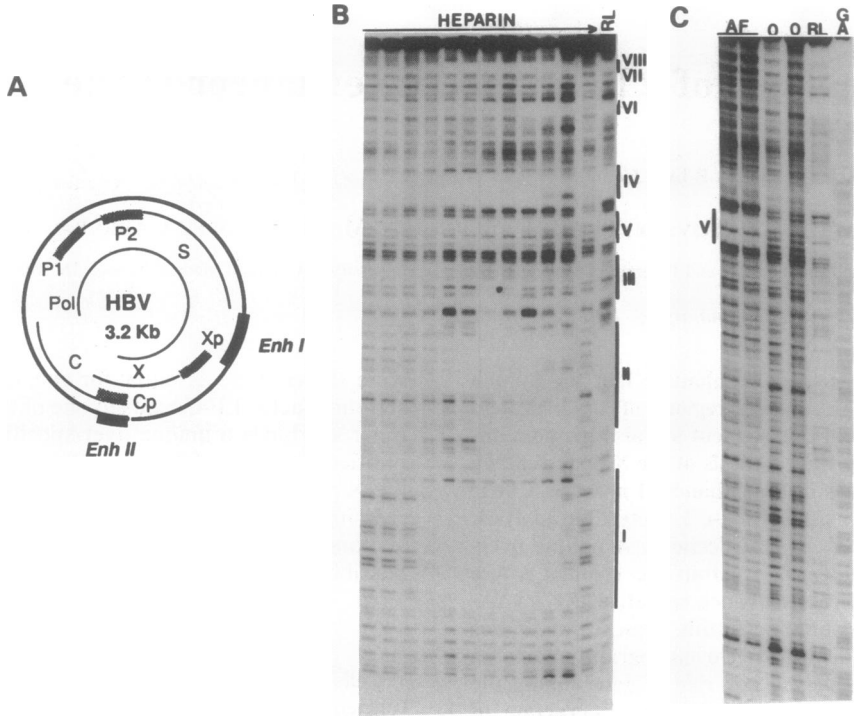

FIG. 1. (*A*) Genomic organization of the HBV DNA. The open reading frames (ORFs) S, C, Pol, and X are shown with the corresponding promoters P1, P2, Cp, and Xp. Kb, kilobases. (*B*) DNase I protection analysis of the enhancer/X promoter complex with liver extracts fractionated through a heparin-Sepharose column. The 5′ end-labeled probe contained sequences from *Hpa* I to *Hpa* II [966–1308 nucleotides (nt)]. Rat liver extracts (RLs) were passed through the heparin-Sepharose column and eluted in the presence of increasing concentration of KCl (0.1–1 M) shown by the arrow. Footprints (FPs) are indicated as I–VIII. (*C*) Oligonucleotide-affinity chromatography. FPV-related heparin-Sepharose fractions obtained above were passed through a FPV-related oligonucleotide-Sepharose column (25) and assayed by DNase I protection assay. 0, Without any protein; AF, affinity-purified and eluted fractions. A/G denotes the sequencing ladder. The FPs seen with RL are similar to those described in *B*.

moterless luciferase gene in plasmid pxp1luc or pxp2luc (20). The first ATG of the X gene contained in the restriction site *Nco* I was destroyed. The nomenclature of mutant plasmids is derived from the FP regions they contain. For instance, p53Sluc contains sequences of FPV and FPIII in front of the SV40 early promoter controlling luciferase gene expression. Plasmids containing designation N are those in which cloned sequences extended up to the *Nco* I site at the X ORF boundary.

Cotransfection with Genes Encoding Transcription Factors. The expression vectors for C/EBP (pMSV-C/EBP or pSV-C/EBP) and Jun (pRS-cJUN) (22, 23) were used in cotransfection studies along with the enhancer/X promoter constructs to assess the role of these factors. In cotransfections, about 2–5 μg of the HBV enhancer/X promoter constructs and variable concentrations of expression vector DNA of a given transcription factor ranging from 50 ng to 10 μg were utilized.

RESULTS

Deletion Mutagenesis of the HBV Enhancer Element I. HBV enhancer element I has been previously shown to exhibit preference for liver cells (6, 7, 14–17). DNA–protein interactions between the enhancer and transcriptional factors have been described previously (6, 14–17). A typical pattern of such interactions using proteins fractionated through a heparin-Sepharose column is illustrated in Fig. 1*B*. We have previously defined these sites of interaction as FPI–FPVIII (14).

To address the functional importance of the interactions, combinations of the regions including defined FPs were generated by PCR-mediated amplification (27). These fragments were then placed in front of the SV40 early promoter controlling the luciferase gene expression. Results of this analysis are described in Fig. 2. Deletion of sequences containing FPVI–FPVIII (p51Sluc) did not have any effect on the enhancer function. The modest 2-fold increase by p51Sluc probably reflects the release of regulatory constraints in the enhancer sequences contained in pEISluc. Plasmids containing FPVIII–FPVI (p86Sluc) or FPVIII–FPIV (p84Sluc) were unable to exhibit enhancer function. Exclusion of sequences

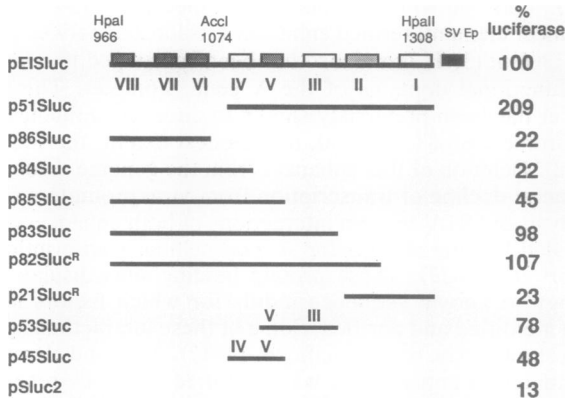

FIG. 2. Functional domains of the enhancer I. Expression of firefly luciferase gene under the transcriptional control of the HBV enhancer deletion mutants and the SV40 early promoter (SV Ep). The structure of the wild-type enhancer and the location of the eight FP regions are shown. The sequences retained in each of the enhancer deletion mutants are indicated by solid lines. Luciferase gene expression was measured in Huh-7 cells as described by deWet *et al.* (19). The levels of enhancer activity by the mutants are expressed as the percentage of light units measured for each of the constructs. The wild-type enhancer value was set arbitrarily at 100%.

contained in FPII and FPI (Fig. 2) also did not seem to affect the enhancer function in several vectors. When these sequences were present in either orientation [p21Sluc (data not shown) or p21Sluc^R], no enhancer activity was observed. However, the sequences included in FPV–FPIII together were efficient in supporting the enhancer function, as shown by retention of ≈80% of its activity in transfections with the plasmid p53Sluc. Plasmid p45Sluc, which contains FPV and FPIV, seems to be only 48% efficient. These results, taken together, define a region represented by FPIII and FPV, which may constitute the principal functional domains of the enhancer and thus may be defined as the "core enhancer sequence." The protein that binds to FPIII or the "EP" element (15) has been identified as EF-C (18, 28). The factor(s) that occupy FPV have not been identified. However, Patel *et al.* (14) have previously identified a liver-specific FP in this region. HeLa extracts failed to show binding at this site (14).

To investigate the factor(s) involved in binding to FPV, we carried out the purification of the proteins by oligonucleotide-affinity chromatography (25). RLNEs were partially purified by passage through a heparin-Sepharose column. The fractions were analyzed by DNase I protection assay using an end-labeled FPV-related oligonucleotide (Fig. 1*C*). FPV-containing fractions were then pooled and purified by oligonucleotide-affinity column chromatography. Only the region corresponding to the previously defined FPV is protected by the purified preparation (Fig. 1*C*).

X Gene Promoter Element. The X gene is located in the HBV genome between 1375 and 1850 nt. A putative promoter activity has been identified by reporter gene assays (10, 11). S1 nuclease mapping analysis defines the mRNA initiation sites at map positions 1239 and 1246 nt (13) immediately downstream of the NF-1 site in FPI (Fig. 3). *In vitro* transcription studies located an additional site of initiation at nt 1288 (29). The functional domains of this promoter, which may overlap with those of the enhancer I, were studied by PCR-mediated mutagenesis. The results of this deletion analysis are described in Fig. 3. Sequences from *Hpa* I to *Nco* I (p81Nluc, 966–1375 nt) or *Acc* I to *Nco* I (p61Nluc, 1074–1375 nt) placed in front of the luciferase gene (promoterless luciferase plasmid, pXP2luc) were equally efficient in producing a high level of luciferase expression. In a previous study we had noted that these sequences were ineffective in transcriptional activation when placed in an opposite orientation (10). The construct p83luc was also inefficient in supporting any transcriptional activity. The plasmid p21Nluc, which includes FPII and FPI and extends to the *Nco* I site, is sufficient to produce a basal level of promoter activity. This activity is comparable to the luciferase expression observed from the SV40 early promoter (pSluc2). Sequences contained in FPII and FPI alone were unable to support promoter activity (p21luc). This plasmid lacked sequences 3′ to the NF-1a binding site marked by the restriction sites *Sph* I to *Nco* I (1239–1375 nt). These observations suggest the importance of sequences that may function as the 5′ leader of X mRNA. Inclusion of the FPIII sequence did not increase the level of promoter activity (p31Nluc). Plasmid p21Nluc^R, in which the FPI, FPII, and the sequences up to *Nco* I are in the reverse orientation, does not retain any promoter activity, consistent with the orientation dependence of promoter domains (30, 31). Taken together, this analysis localizes the domains of a basal promoter for the X gene between nt 1168 (−201) and 1323 (*Alu* I site) and includes multiple protein binding domains represented by FPII and FPI. Interestingly, the promoter domain lacks a canonical TATA box. In the context of the genome the X promoter is under the regulatory constraints of the enhancer, which displays a preference for liver cells as seen with p61Nluc (Fig. 3). Expression of X gene under the control of upstream enhancer/promoter complex has been observed to be restricted to liver cells (ref. 10; A.S., unpublished results).

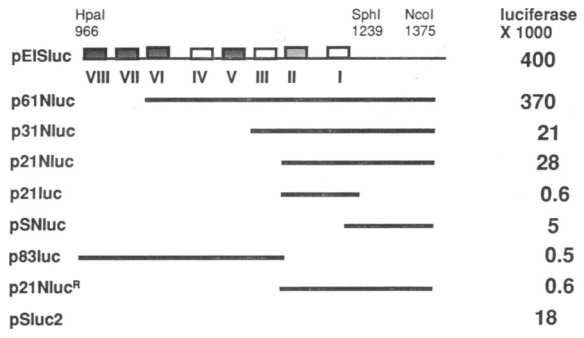

FIG. 3. X promoter domains. PCR-mediated deletion mutants were generated and cloned in front of the luciferase gene and assayed in Huh-7 or HepG2 cells. The sequences retained in the mutants are shown as solid lines. The luciferase expression is expressed as light units obtained by each cell lysate. pSluc2 contains the SV40 early promoter.

Binding of Purified Factors. The enhancer/promoter complex contains several sequence motifs with homology to purified and well-characterized factors. These include C/EBP, AP-1 (fos/jun), CREB/ATF, eH-TF, and NF-1 (Fig. 1*B*) (30, 32–34). Most of these interactions reside in the region of FPII. The pattern of DNase I protection in this region obtained from heparin-Sepharose fractionation of RLNE clearly indicates that multiple proteins, eluting at different salt concentrations, bind at FPII (Fig. 1*B*). We have employed several of these purified factors in binding studies and demonstrate their interaction to sequences in the enhancer/promoter element (Fig. 1*B*).

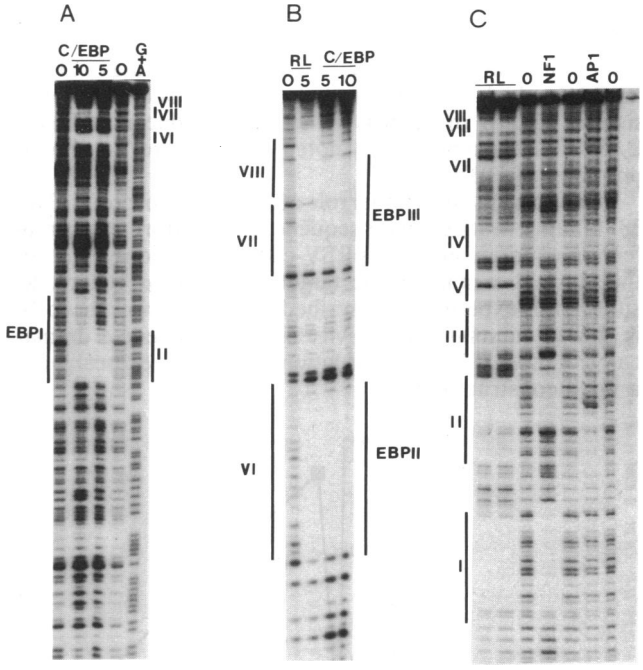

FIG. 4. Binding of purified factors. (*A*) FP analysis of the enhancer I fragment *Hpa* II–*Acc* I (1074–1308 nt) labeled at the *Hpa* II (1308 nt) end in the polylinker. Binding reaction mixtures contained the indicated amounts of r-C/EBP. EBPI denotes the C/EBP binding site that overlaps with FPII. (*B*) DNase I protection analysis using the *Hpa* I–*Acc* I (966–1074 nt) fragment of enhancer I with r-C/EBP. EBPII and EBPIII are the C/EBP binding sites that overlap with FPVI and FPVII/FPVIII, respectively. (*C*) Binding of partially purified factors NF-1 and AP-1 assayed by DNase I protection assay. 0, No protein. Labels are as indicated in Fig. 1*B*.

C/EBP, a liver-enriched factor, binds to enhancer sequences (2, 34). Two of these C/EBP binding sites have been reported by Landschulz *et al.* (21) using C/EBP synthesized in *E. coli* (r-C/EBP). To demonstrate that C/EBP occupies these sites in RLNE, we utilized the heat-stable property of C/EBP (2). RLNE was heated to 70°C for 5 min prior to addition to the binding reaction. Footprinting patterns show the binding of a heat-stable protein at FPII and FPVI (not shown). Competition with the CCAAT oligonucleotide (2), which has binding affinity for C/EBP, was also used to confirm the C/EBP interactions. Using a partially purified r-C/EBP, we have discovered a third site of interaction overlapping FPVII and FPVIII in addition to the previously defined sites (Fig. 4 *A* and *B*). This interaction also appears with RLNE following heat treatment.

Binding of partially purified AP-1 can be observed to a sequence motif known as phorbol 12-myristate 13-acetate-inducible responsive element or TRE (33) (Fig. 4*C*). NF-1, purified from HeLa cells, binds at three sites in the enhancer (Fig. 4*C*). These binding sites are consistent with those described previously (15). In the liver, an NF-1-like protein termed NF-1L (35) probably binds at these sites.

Trans-Activation of the X Promoter. To assess the contribution of C/EBP and AP-1 binding to the X gene promoter, we carried out cotransfection experiments with expression vectors for these transcriptional factors along with the vectors containing domains of the promoter. When p21Nluc (includes FPII and FPI) was used in cotransfections along with the C/EBP vector [pSV-EBP (36) or pMSV-EBP (22)], trans-activation was observed (Fig. 5*A*). In contrast to the stimulation of X-promoter activity by C/EBP, repression of enhancer activity similar to the one shown with p31Nluc (Fig. 5*A*) has been observed in the presence of overexpressed C/EBP in cotransfection experiments (ref. 37; A.S., unpublished results). This can be explained by the observation that interactions with r-C/EBP extend into the FPIII region (Fig. 4*A*). Such an extended binding, which occurs when C/EBP is present in larger amounts, may preclude binding of factors to FPIII, which we show are crucial to enhancer function. A similar pattern of repression at higher concentrations of C/EBP has been previously noted with the core/pregenomic promoter of HBV (36).

To confirm the functional role of AP-1 (jun/fos; refs. 33 and 38) binding to its cognate sequence, we performed cotransfection experiments with pRSV-cJun expression vector and the plasmid containing the target sequence (p21Nluc). Results of this experiment, described in Fig. 5*B*, show that c-Jun is able to stimulate the activity of HBV sequences dramatically. A modest 2-fold stimulation was seen previously using a v-*jun* construct (15).

DISCUSSION

We have conducted a functional analysis of the enhancer/promoter complex, a sequence with multiple domains. This was carried out by generating deletion mutants and analyzing their subsequent expression following transfection. DNase I protection assays were performed with several purified factors to demonstrate this binding to FPs previously identified with crude nuclear extracts. Results from these studies can be summarized as follows: (*i*) PCR-mediated deletion analysis defines functionally important DNA–protein interactions of the enhancer element I and those of the X promoter, (*ii*) cotransfection studies with cDNAs of the transcriptional factors display trans-activation, and (*iii*) a liver-specific factor(s), which we have purified and tentatively called hepatitis B liver factor (HBLF), binds to FPV. This binding region is shown by deletion analysis to play a key role in overall enhancer function in conjunction with the interactions of EF-C at FPIII. EF-C was first recognized in F9 embryonal carcinoma cells and shown to bind a sequence motif in the polyoma virus enhancer element C (27). Thus, interaction between ubiquitous (EF-C) and liver-specific factors (i.e., HBLF) may play a dominant role in controlling the enhancer function.

The region defined as "E" element, or FPII in this analysis, was shown previously to be an essential element of the enhancer (15). Genetic studies described here (Fig. 2) clearly show that deletion of FPII has little, if any, effect on the enhancer function. On the other hand, this region constitutes an important component of the X promoter. However, interactions in this region may contribute to the overall efficiency of enhancer function in the context of the enhancer/promoter complex during natural infection.

The observed trans-activation with c-Jun (Fig. 5*B*) coupled with the demonstration of AP-1 binding to the enhancer/promoter element (Fig. 4*C*) implicates involvement of the protein kinase C signal transduction pathway in the transcriptional control of HBV genes. The inhibition of X promoter activity observed in cotransfections with higher concentrations of either C/EBP or c-Jun may be due to squelching (39, 40). In this respect, the activation domains of C/EBP recently identified may be implicated in interactions with other cellular factors (41).

On the other hand, repression of the whole HBV enhancer activity in the presence of C/EBP, which has been noted previously (37) and shown here for p31Nluc (Fig. 5*A*), could result from interference of binding of EF-C to FPIII. This conclusion is based on the following observations: (*i*) in the presence of higher concentrations of r-C/EBP an extended FP was observed (Fig. 4*A*) and (*ii*) activation of the X

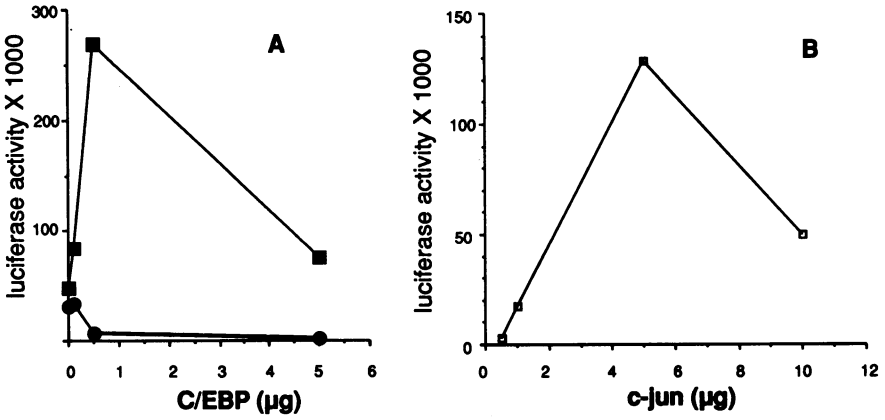

FIG. 5. Cotransfections with C/EBP and c-Jun expression vectors. (*A*) Transient cotransfections in Huh-7 cells included a constant amount (2 μg) of plasmid p31Nluc (●) or p21Nluc (■) and the indicated amounts (μg) of C/EBP encoding vector pMSV-C/EBP (22) or pSV-C/EBP (36). (*B*) Cotransfection with pRSV-cJun vector with p21Nluc (□). Luciferase activity is represented as light units.

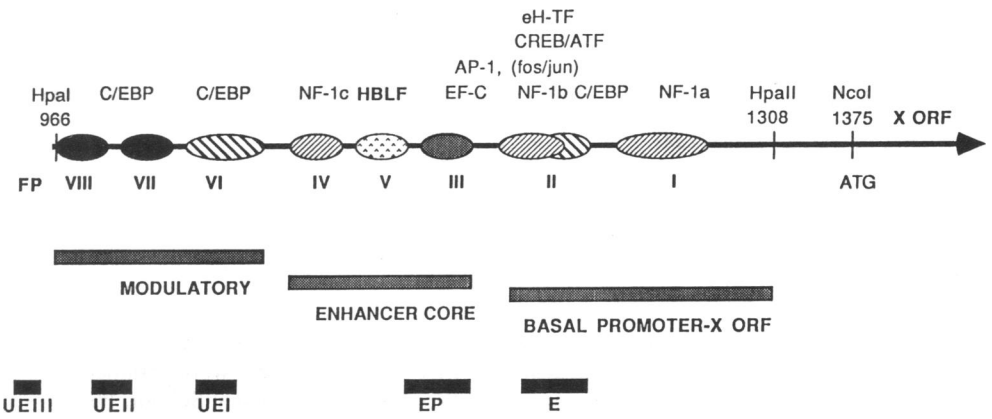

FIG. 6. Functional domains of the HBV enhancer I. Functional domains of the enhancer are described as modulatory domain, core enhancer, and basal promoter for the X ORF. Terminology used by Faktor *et al.* (15) is shown as E, EP, UEI–UEIII.

promoter (p21Nluc) was achieved when the sequences contained within FPIII were removed.

Based on the genetic analysis described above the following model is proposed (Fig. 6). In this model, the sequences previously identified as active enhancer (966–1308 nt) are dissected into three domains: (*i*) a modulatory domain represented by sequences included in FPIV–FPVIII, (*ii*) a core enhancer domain represented by interactions at FPV and FPIII, and (*iii*) a basal X promoter domain containing binding sites for multiple factors defined by FPII and FPI.

Enhancer activity in the context of the HBV genome must require full participation of all possible DNA–protein interactions to produce the regulatory levels of expression of HBV genes. In view of the complex nature of DNA–protein interaction as evidenced by footprinting patterns, a synergistic interaction between each domain may be necessary to achieve the required levels of regulation.

Recently a second enhancer element in the HBV genome was mapped within the core/pregenomic promoter (5). It has been termed enhancer II as opposed to the previously defined enhancer, which is now called enhancer I. It appears that whereas enhancer I mostly affects the activity of the core/pregenomic and the X promoters (7, 8, 36), enhancer II regulates the activity of the S promoters (42, 43). The presence of two enhancer-like sequences in the small genome of HBV is quite intriguing but, given the complexity of molecular features of the HBV DNA, these control elements may interact with each other to give rise to a tightly regulated viral gene expression and replication. Synthesis of the cellular trans-acting proteins in liver cells may ultimately be the determining factor in the control of HBV gene expression and course of infection.

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