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Interactions between Nuclear Factors and the Hepatitis B Virus Enhancer

NITA U. PATEL,[1] SHAHID JAMEEL,[1]† HARRIET ISOM,[2] AND ALEEM SIDDIQUI[1]*

Department of Microbiology and Immunology and Program in Molecular Biology, University of Colorado Medical School, Denver, Colorado 80262,[1] and Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033[2]

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We have previously established that the tissue-specific activity of the hepatitis B virus (HBV) enhancer is mediated by *trans*-acting cellular factors. Here we have studied in vitro the interactions between the HBV enhancer DNA and cellular factors present in nuclear extracts from both liver and nonliver cell types. The results presented in this study imply the involvement of several distinct, ubiquitous, and liver-specific cellular factors with the HBV enhancer. Sequence analysis of the binding sites for these proteins on HBV DNA showed homologies to sequence motifs known to bind other previously characterized and purified transcription factors including CAAT/enhancer-binding protein. Thus, all of these binding sites may function in concert to activate liver-specific transcription of HBV genes from their respective promoters.

Human hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is a small DNA virus that exhibits marked liver tropism (for reviews, see references 11 and 29). Such hepatotropic expression may be due to preferential attachment and entry into hepatocytes, liver-specific viral regulatory elements, or both. One such HBV regulatory element that has generated interest is the viral enhancer (37, 40). Transcriptional enhancers, including the HBV element, are *cis*-acting regions of DNA that can potentiate transcription from autologous and heterologous promoters in a relatively position- and orientation-independent manner (16, 37, 40). The HBV enhancer element is strategically placed (Fig. 1A) 3′ to the surface antigen gene and 5′ to the core and X antigen genes and is included in the coding frame of the putative polymerase gene. Such a location makes it plausible for the enhancer to modulate activity of the core and X promoters located downstream and the surface antigen promoter(s) located upstream. Requirement of the enhancer for efficient expression of S antigen and X genes has been previously demonstrated (2, 16, 38). Furthermore, it is the only enhancer, to our knowledge, whose sequences are part of mature transcripts, in this case the 2.1-kilobase surface antigen mRNA and the 3.5-kilobase RNA species including the pregenomic RNA (for a review, see reference 11).

It has been previously shown by means of gene transfer experiments that the HBV enhancer exhibits cell type specificity for human hepatoma cells and that this specificity is due to *trans*-acting factors (16, 37). There is substantial evidence for the involvement of a variety of proteins in the regulation of transcription (12, 20, 28, 32, 39). The binding sites for such *trans*-acting protein factors have been mapped on a number of viral and cellular enhancers (3, 29, 32–35, 41). It is now becoming increasingly evident that the enhancer-mediated transcriptional activation and tissue specificity involve interactions between conserved sequence motifs on the DNA and closely related cellular factors. The mechanisms for such a regulatory control, however, have yet to be established. A cassette-shuffling model has been proposed to explain the substantial overlap seen between various enhancers in terms of sequence motifs and the factors that bind to them (34).

Because of the host and tissue specificities of the HBV enhancer, we set out to identify and characterize in detail the nature of the protein-DNA interactions between *trans*-acting cellular factors and the HBV enhancer element. Using DNase I protection analysis, we define here the binding sites of both liver-specific and ubiquitous factors on the enhancer element and discuss their possible significance in HBV gene expression.

MATERIALS AND METHODS

Cell lines and extracts. Huh-7 (25) and HepG2, human hepatoma cell lines that are negative for HBV sequences, and HeLa, a human cervical carcinoma cell line, were used. Nuclear extracts were prepared from HeLa, Huh-7, rat liver, and spleen tissues. Cultured cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (75 U of penicillin and 50 mg of streptomycin per ml). Nuclear extracts from HeLa and Huh-7 cells were prepared by the method of Dignam et al. (7). Extraction of nuclear proteins from rat liver and spleen was carried out by the method of Johnson et al. (18). Protein concentrations were determined by using the Bio-Rad protein assay kit and were typically between 3 and 12 mg/ml. Extracts were stored at −70°C. Luciferase assays were carried out by the method of de Wet et al. (6). Cell transfections were by the $CaPO_4$ method (14). Plasmid pSV2CAT (13) was used as a control plasmid to normalize the transfection values for the differential uptake by different cell lines. Methods of isolation, propagation, and immortalization of rat primary hepatocytes to generate cell line CWSV1 have been previously described (42).

Plasmids and preparation of labeled probes. The 342-base-pair *Hpa*I-*Hpa*II (from nucleotide [nt] 966 to 1308) fragment of HBV DNA (Fig. 1) was isolated and subcloned into plasmid pSP65 at its *Hin*dII site to produce the plasmid pEnh. Radiolabeled probes were prepared by digesting pEnh

* Corresponding author.
† Present address: UNIDO International Center for Genetic Engineering and Biotechnology, New Delhi-110067, India.

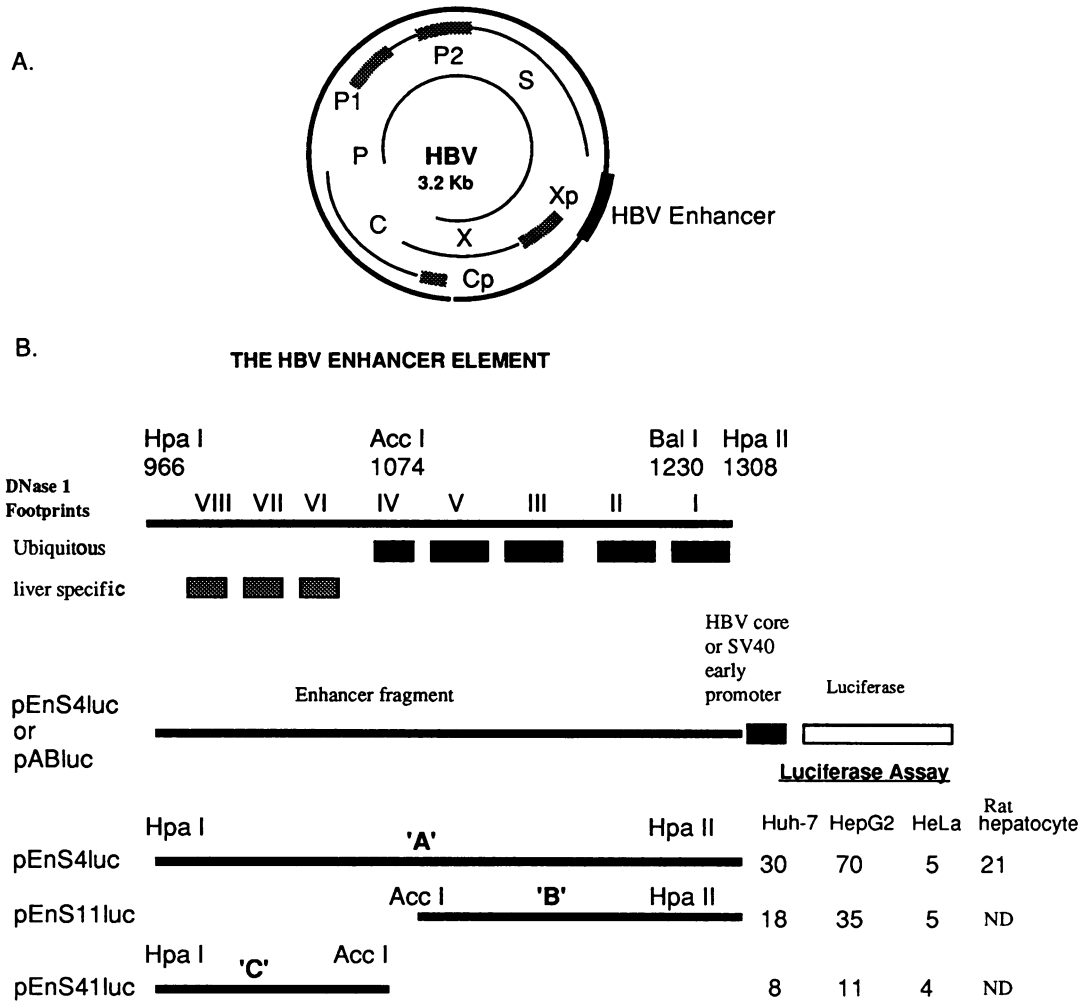

FIG. 1. (A) HBV genome with open reading frames (S, C, X, and P) and promoters (P1, P2, Xp, and Cp). (B) Enhancer region showing the distribution of various protein-binding regions. Expression of firefly luciferase gene under the transcriptional control of either the HBV core gene promoter, pABluc (*AvaI-BglII*, nt 1466 to 1987), or the SV40 early promoter region (41) and the HBV enhancer fragments A (pEnS41uc), B (pEnS11), and C (pEnS41) located upstream. The levels of luciferase expression are presented as fold stimulation expressed as light units above the background levels obtained with either the HBV core gene or the SV40 early promoter alone without the HBV enhancer. HepG2 and Huh-7 cells were derived from human hepatomas, and CWSV1 cells were derived from primary rat hepatocytes (42). ND, Not done.

at either the *Bam*HI or *Hin*dIII site of the SP65 polylinker, dephosphorylating the 5′ end with calf intestine alkaline phosphatase, and end labeling the 5′ ends with [γ-^{32}P]ATP (>4,000 Ci/mM; ICN Pharmaceuticals, Inc.) by T4 polynucleotide kinase (United States Biochemicals). The DNA was then digested with another restriction enzyme to produce fragments labeled at one terminus. Smaller labeled fragments were prepared by cutting at the *Acc*I site (nt 1074) within the HBV enhancer followed by 5′-end labeling. The probes were purified and eluted from 6% native polyacrylamide gels. The specific activities typically ranged between 3 $\times$ 10^4 and 1 $\times$ 10^5 cpm/ng of DNA.

DNase I protection analysis. For DNase I protection analysis (10), 5 to 10 pmol of probe (about 50,000 cpm) was mixed with 1 μg of poly(dI · dC) and an appropriate amount of nuclear extract (0 to 150 μg) in a final volume of 50 μl of binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM dithiothreitol). After binding on ice for 15 min, 50 μl of a solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was added to each tube. After a 1-min incubation at room temperature, an appropriate amount (empirically determined) of freshly diluted DNase I was added and the digestion was carried out for exactly 1 min at room temperature. The digestion was quenched with 90 μl of stop buffer (0.2 M NaCl, 0.03 M EDTA, 1% sodium dodecyl sulfate, 100 μg of tRNA per ml). The samples were extracted once with phenol-chloroform, and the aqueous phase was precipitated with ethanol. The pellets were dried and suspended in 8 μl of loading buffer (0.1 M NaOH–formamide [1:2, vol/vol], 0.1% xylene cyanol, 0.1% bromophenol blue). After heating for 5 min at 100°C, 4 μl of each sample was subjected to electrophoresis, as described earlier. Marker sequencing ladders were generated by using homologous fragments by the method of Maxam and Gilbert (23).

For footprint competition, a 50- to 100-fold molar excess of either homologous or heterologous fragments or oligonucleotides was mixed with the corresponding HBV enhancer

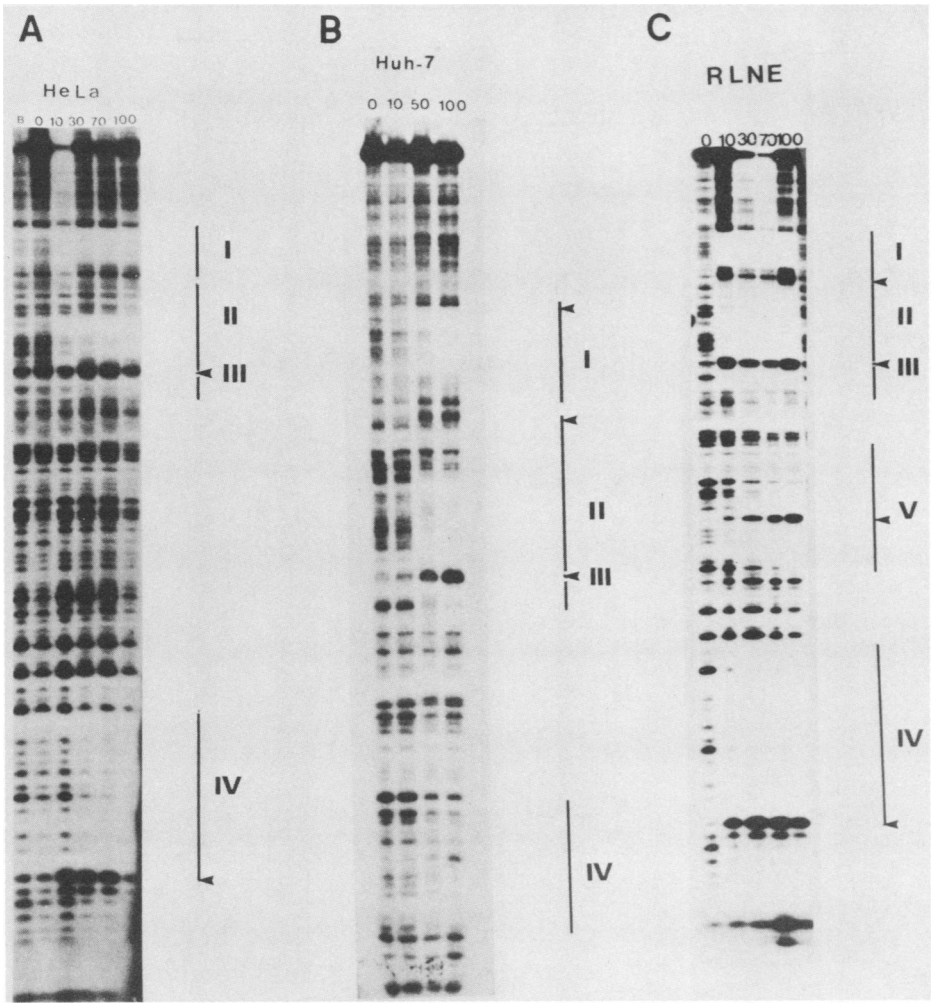

FIG. 2. DNase I protection analysis of fragment B (*Acc*I-*Hpa*II, nt 1074 to 1308) of HBV enhancer by factors contained in the nuclear extracts from HeLa cells (A), Huh-7 cells (B), and RLNE (C). Fragments were 5′ end labeled at the *Acc*I site (coding strand). |, Region of DNase I protection; ◀, hypersensitive site. The amounts of nuclear proteins used are given in micrograms (0 to 100 μg).

probe fragments and incubated with rat liver nuclear extract (RLNE).

RESULTS

The HBV enhancer has been previously mapped on the genome from nt 966 to 1308 (37, 40). To compare the activities of the HBV enhancer among the various liver and nonliver cells, we used the firefly luciferase reporter gene in a transient scheme of expression (6). The luciferase gene (*luc*) expression plasmids (Fig. 1B) and enhancer sequences contained either the simian virus 40 (SV40) early promoter (41) (pEnS41uc) or the HBV core gene promoter (pABluc). The relative levels of induction of *luc* gene activity in the liver-derived cell lines (Huh-7, HepG2, and rat hepatocytes) and nonliver HeLa cells are shown in Fig. 1B. The levels of induction of luciferase activity with fragment A ranged from 30- to 70-fold above the values obtained with either the luciferase expression plasmid pSV232luc (with the enhancer-less SV40 early promoter) (6) or pABluc (the HBV core and pregenomic promoter) in liver-derived cell lines but were only fivefold greater in HeLa, establishing its cell type-specific enhancer activity. The *luc* activity with fragment B showed approximately similar levels of stimulation, whereas fragment C was insufficient for full enhancer activity (Fig. 1B). These data are in agreement with data from previous studies from this and other laboratories (1, 16, 37). All transfection experiments include an independent plasmid containing an SV40-driven chloramphenicol acetyltransferase (13) gene that was used to monitor transfection efficiency (data not presented). Of interest in the present study is the demonstration of enhancer function in CWSV1, a cell line derived from rat hepatocytes that have been immortalized by SV40 T antigen (42). In contrast to our previous results, in which HBV enhancer function was shown to be restricted to human liver cell lines, these results clearly show that a cell line derived from rat hepatocytes can support enhancer activity. Further support for this observation is provided by the recent success of replication and expression of the HBV genome in transgenic mice (8).

Multiple factors bind to the HBV enhancer. Because of the observed liver cell type specificity, DNase I protection analysis (10) was carried out to define the binding sites of cellular factors with the enhancer element. For DNase I

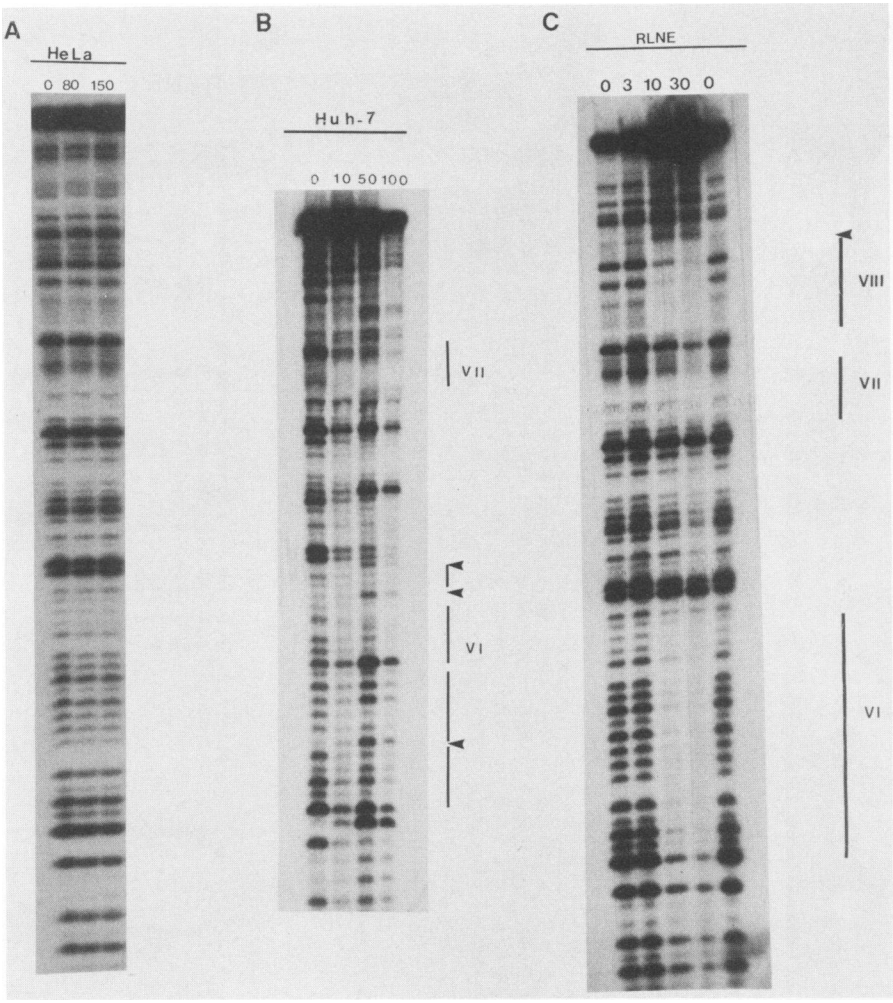

FIG. 3. DNase I protection analysis of fragment C (*Hpa*I-*Acc*I, nt 966 to 1074). Nuclear extracts used were from HeLa cells (A), Huh-7 cells (B), and RLNE (C). Symbols are as defined in the legend to Fig. 2.

protection analysis, the 342-base-pair *Hpa*I-*Hpa*II (nt 966 to 1308) fragment A (Fig. 1) was subdivided into fragments B (*Acc*I-*Hpa*II, nt 1074 to 1308) and C (*Hpa*I-*Acc*I, nt 966 to 1074) by using the convenient restriction site *Acc*I (nt 1074). All three fragments (A through C) were subsequently used as probes. All DNase I protection experiments were carried out in the presence of excess poly(dI · dC). The digests were then fractionated with homologous nucleotide sequence ladders on denaturing polyacrylamide gels. Treatment of the extracts with proteinase K or heat at 99°C for 15 min prior to DNase I digestion abolished all footprints (data not shown). The footprint analysis of fragment B (*Acc*I-*Hpa*II, nt 1074 to 1308) incubated with nuclear extracts prepared from HeLa, Huh-7, and RLNE is shown in Fig. 2. At least four distinct footprints, designated FP-I to FP-IV, were observed within the HBV enhancer fragment B on the coding (Fig. 2) as well as on the noncoding (data not shown) strand with all the extracts used. An additional footprint, FP-V, was also observed only in the presence of RLNE extract (Fig. 2C). This footprint, however, was weak with Huh-7 extracts. Thus it is difficult at this time to suggest that this interaction is liver specific. The nucleotide sequence of each of these protected regions and its relative position on the HBV enhancer element are summarized in Fig. 6. The five footprint regions map between nucleotide sequence positions: 1208 and 1238 (FP-I), 1168 and 1204 (FP-II), 1150 and 1164 (FP-III), 1087 and 1116 (FP-IV), and 1120 and 1137 (FP-V). Most of the protected regions are flanked by hypersensitive sites. Similar, if not identical, DNase I footprint patterns were obtained for FP-I to FP-V with nuclear extracts from both liver- and non-liver-derived cells or tissues (Fig. 2, and see Fig. 4), indicating the ubiquitous nature of the factors binding to the various sequence motifs contained in this fragment.

DNase I protection analysis of fragment C (*Hpa*I-*Acc*I, nt 966 to 1074) revealed at least three principal areas of interaction with liver extracts (FP-VI through FP-VIII). The FP-VIII interaction is not immediately evident with Huh-7 extracts. When HeLa cells were used, we did not observe any noticeable binding in this fragment (Fig. 3A). FP-VI is protected between nucleotide positions 1017 and 1045, FP-VII is protected between nt 993 and 1009, and FP-VIII is protected between nt 971 and 991. Each region of protection was again flanked by hypersensitive sites. Interestingly, with the Huh-7 extract (Fig. 3B), the protected region of FP-VI contained several internal hypersensitive sites compared with the FP-VI of RLNE (Fig. 3C), indicating the binding of at least two or more factors to this region. Alternatively,

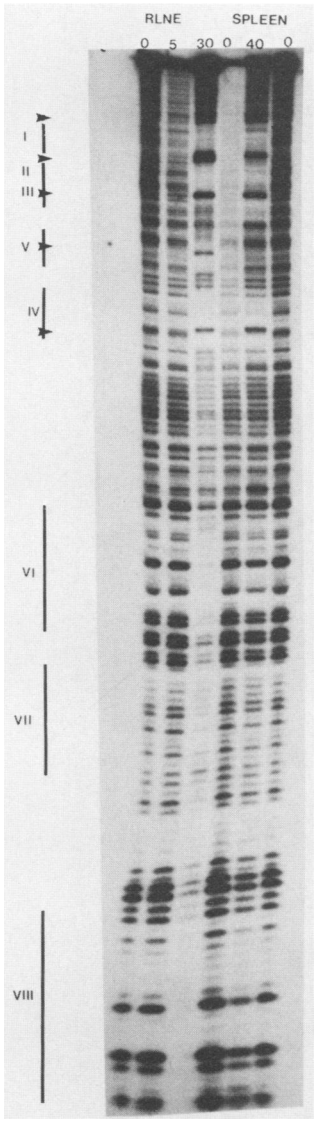

FIG. 4. DNase I protection analysis of the entire HBV enhancer fragment A (*Hpa*I to *Hpa*II, nt 966 to 1308) by factors contained in RLNE or spleen nuclear extracts. Lane 0 (middle) was overdigested with DNase I in the upper portion of the autoradiogram. Symbols are as defined in the legend to Fig. 2.

several factors present in RLNE may also bind to this region but with overlapping target sites. Thus, the factors binding to the nucleotide sequences in FP-VI to FP-VIII appear to be liver specific.

We next subjected the entire enhancer fragment A to DNase I protection analysis. The footprinting pattern with fragment A (the coding strand) was similar to that seen with subfragments B and C, demonstrating that dissection of the enhancer fragment did not have any noticeable effect on the overall footprinting pattern (Fig. 4). Footprints FP-VI to FP-VIII were not observed in the presence of spleen nuclear extracts, thereby reaffirming the liver-specific nature of the factors. The general footprinting patterns with RLNEs are noticeably clear compared with those of human hepatoma cell line extracts, perhaps indicating the abundance of these proteins in tissues compared with their abundance in cell lines (Figs. 2 through 5).

A sequence analysis of the DNase I-protected regions (FP-I to FP-VIII) within the enhancer element revealed homologies to several known sequence motifs. These are the nuclear factor (NF)-1 binding site (FP-I), the SV40 enhancer core element (FP-II and FP-VI), the polyomavirus enhancer factor EF-C element (FP-III), and the hepatocyte nuclear factor-1 (HNF-1) sequence (FP-VI). By using some of the sequences described above as competitors in DNase I protection experiments, we show here that HBV enhancer element may have the same factors as elements found in other enhancers and promoters (Fig. 5). This is shown by binding competitions with unlabeled oligonucleotide sequences and fragments corresponding to the sequence motifs. While this study attempts to demonstrate that distinct factors may be binding to the various sequences on the HBV enhancer element (by competitions, heat stability, and heparin-Sepharose column chromatography), the identity of the factor(s) specific to the sequence motifs remains to be determined.

A palindrome sequence (5′-TGGCCATAGGCCA-3′) in the protected region FP-I (between nt 1223 and 1236) shows considerable homology to the binding site for NF-1. NF-1, a protein originally purified from HeLa cells (30), has recently been assigned a multifunctional role, i.e., adenovirus DNA replication and transcription (24). It has recently been shown to be similar to the transcription factor responsible for selective recognition of eucaryotic promoters containing the sequence CCAAT (19). When we used a multimeric oligomer containing the NF-1 binding site (30), we observed no competition at the FP-I site (Fig. 5A). Further, the inability of HBV enhancer DNA to displace purified NF-1 from its authentic binding site in a competition assay (unpublished results of R. M. Gronostajski, Ontario, Canada, and J. Oikarinen, University of Oulo, Finland) indicated that this sequence, although it shows no binding to the NF-1 protein, may be interacting with an NF-1-like family of proteins (15, 20). Ben-Levy et al. (1) recently demonstrated that NF-1 does bind to the sequences of this footprint. However, the purification of these sequences was performed by using an affinity column containing an NF-1-like sequence derived from hepatitis B surface antigen promoter region, and that may explain the different results obtained here.

DNA sequences included in FP-II and FP-VI are homologous at 7 and 6 base pairs, respectively, to the 8-base-pair SV40 enhancer core element 5′-GTGGTTTC-3′(19). This region of protection is effectively displaced by competition with the SV40 enhancer-promoter fragment (Fig. 5B). A purified liver-specific factor, C/EBP (18), has recently been shown to bind to this sequence (22) in FP-II and FP-VI. As mentioned earlier, FP-VI also contains the C/EBP binding site, and juxtaposed to this sequence is the sequence with homology to the HNF-1 sequence motif (5). The oligonucleotide containing the HNF-1 binding sequence removes this footprint (Fig. 5C), suggesting that HNF-1 is required for the C/EBP binding. In fact, our recent preliminary data on point mutations in this region also lend support to this notion (N. U. Patel, unpublished data). Another sequence (CTGAC GCA), which is a part of FP-II, has considerable homology to the AP-1/jun binding site (20). Ben-Levy et al. (1) identified this sequence in the enhancer to be 12-*O*-tetradecanoylphorbol-13-acetate inducible. Immediately adjacent to FP-II, with an internal hypersensitive site, is FP-III, which bears perfect consensus to the polyomavirus EF-C factor-binding sequence and has been shown to be the target site

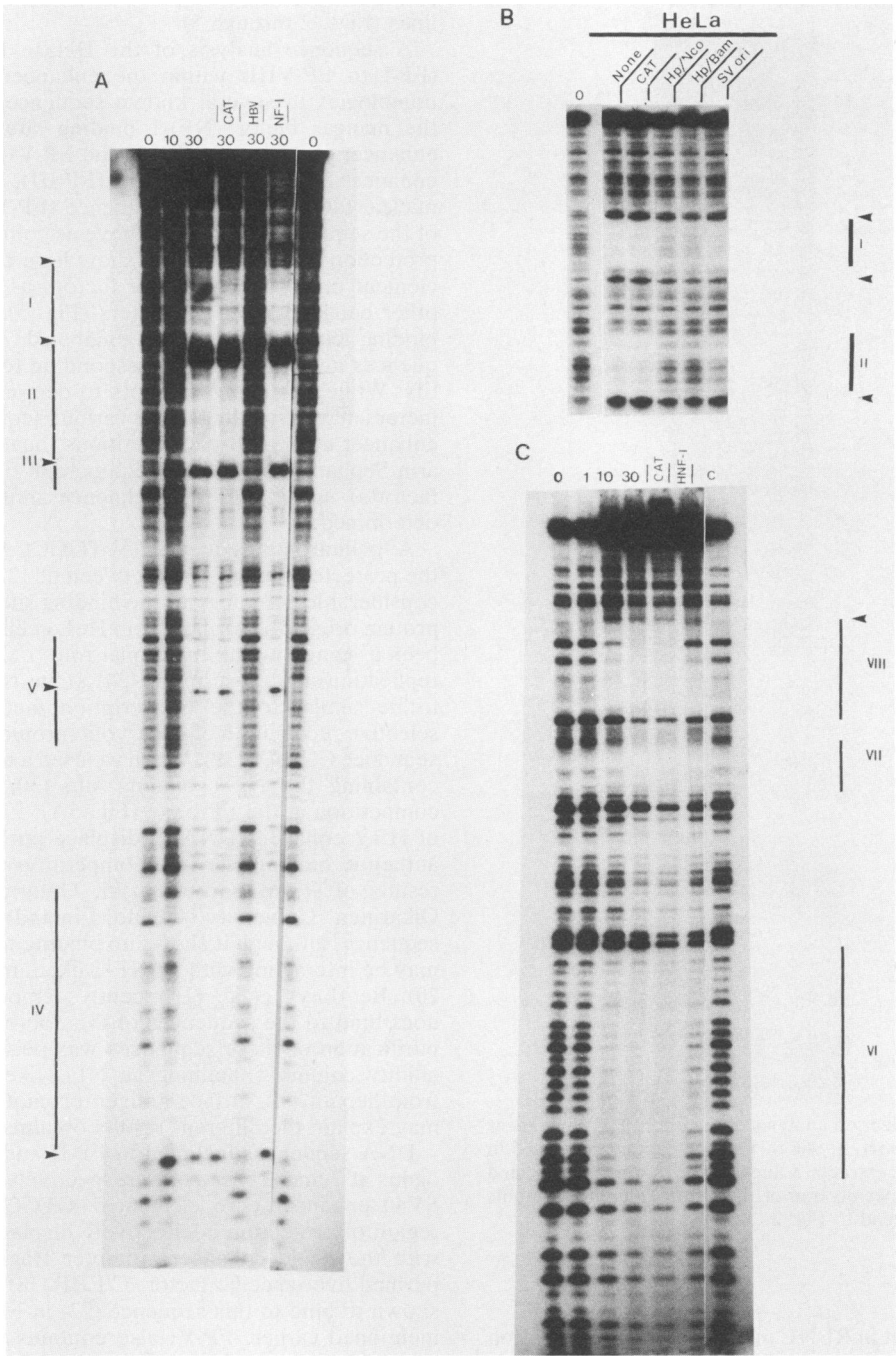

FIG. 5. Footprint competition of fragment B (A and B) and fragment C (C) with unlabeled oligomers or fragments in the presence of nuclear extracts from HeLa cells and RLNE. Competitors included no competitor DNA (none), the bacterial chloramphenicol acetyltransferase gene fragment used as a nonspecific control (CAT), HBV enhancer fragment *Acc*I-*Hpa*II (HBV), a multimeric oligomer (30) (NF-1), and SV40 enhancer sequence *Puv*II-*Hin*dIII (41) (SV ori), an oligomer containing the sequence motif that binds to the protein HNF-1 5′ATTAAC-3′ (5) (HNF-1), the unlabeled fragment C (*Hpa*I-*Acc*I) of the HBV enhancer (C), and *Hpa*I-*Nco*I (nt 966 to 1375) and *Hpa*I-*Bam*HI (nt 966 to 1403) HBV enhancer sequences including the X promoter. Each reaction contained a 100-fold molar excess of unlabeled competitors. Symbols are as defined in the legend to Fig. 2.

for this factor (26). So far, sequence comparisons of footprint regions FP-VII and FP-VIII have not revealed any strong homologies to any previously characterized sequence. Studies are under way to determine the identities of these factors.

DISCUSSION

The tissue-specific activity of the HBV enhancer has been well established by gene transfer methods (16, 37; Fig. 1). We have also shown by in vivo competition experiments that

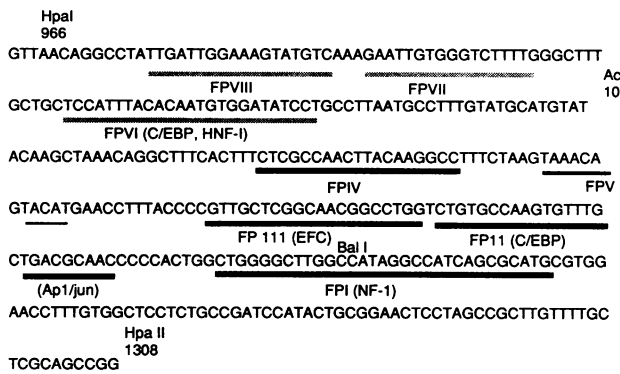

FIG. 6. Summary of the DNase I-protected regions represented by FP-I to FP-VIII. The boundaries of liver- and non-liver-specific protected regions including the coding and noncoding strands are indicated by dotted and solid boxes, respectively. Sequences showing homology to previously characterized consensus motifs are NF-1, C/EBP, EF-C, HNF-I, and AP-1/jun.

trans-acting cellular factors are important for this activity (16, 17, 21, 22, 36). To define and begin to understand such cellular factors, we have studied in vitro the interactions between various cellular extracts of both liver and nonliver cell origins and the HBV enhancer DNA. We show here by means of DNase I protection (footprinting) analysis that the HBV enhancer DNA contains binding sites for multiple cellular factors, some of which appear to be ubiquitous to all cell types while others are specific to liver cells. Interestingly, in the present analysis with the crude nuclear extracts, the target sites for these liver-specific factors all appear to be restricted to fragment C, while the target sites for ubiquitous factors are restricted to fragment B. However, we have found that fragment B alone is capable of conferring both enhancer and cell-type-specific activity, indicating that the fragment contained all the necessary information for mediating HBV enhancer activity (Fig. 1B). An 83-base-pair sequence contained within this fragment, which will include our FP-II and FP-III, was recently shown (1) to produce enhancer activity, although at levels lower than those of fragments containing all five footprints. It is entirely possible that some footprints obtained with fragment B result from binding of similar proteins from nonliver extracts (HeLa or spleen tissue), but these interactions do not promote transcription in nonliver cells (Fig. 1B) (16). For example, C/EBP (22) from RLNE produces FP-II, whereas a similar factor, EBP-1, recently isolated from HeLa cells (4), which has been shown to bind to the SV40 enhancer core element, may have produced FP-II from HeLa extracts.

In contrast to earlier reports, (1, 17, 21, 36), the present study identifies several additional protein binding regions on the functional enhancer element. These regions show striking homologies to known sequence motifs. The DNA sequence specificities of at least two recently purified liver factors show homology to the sequences contained in FP-II and FP-VI. These are C/EBP (18) and HNF-1 (5). Landschulz et al. (22) have shown binding of C/EBP to two regions of the HBV enhancer, called FP-II and FP-VI in this study. We show here that an HNF-1-specific oligomer does compete with FP-VI (Fig. 5B). Moreover, a mutation (Patel, unpublished results) in the HNF-1 sequences abolishes the entire FP-VI, suggesting that a synergy between these factors and perhaps others is required to facilitate binding to the FP-VI region. This result can be confirmed only by demonstrating binding with HNF-1 and C/EBP proteins.

Factor(s) binding to FP-I appears to be ubiquitious. Although its target sequence has close homology to the NF-1 factor, neither an NF-1 sequence oligomer (Fig. 5A) nor a partially purified NF-1 seems to bind to FP-I. The factor(s) binding to these region may be related to the NF-1-like family of proteins (17, 27, 31). Factor II binds to HBV DNA between nt 1180 and 1203. Present within this region is the sequence 5'GTGTTTG-3', which bears close homology to the SV40 enhancer core octamer (Fig. 6). This sequence is found in a variety of viral and cellular enhancers (4, 20, 41) and is shown by point mutations and deletion of the entire sequence to be critical for enhancer activity.

The nucleotide sequences within FP-III and FP-IV are 86% homologous (10 of 12 nt match). These regions on HBV DNA show homology to the binding site of EF-C, a factor identified in HeLa and undifferentiated F9 embryonal carcinoma cells, which binds to the polyomavirus C enhancer element (26). It remains to be seen whether EF-C or a distinct, but closely related, protein is responsible for this binding.

The functional tissue specificity of the HBV enhancer seen in vivo cannot be attributed to any specific interaction at this time. Site-directed mutagenesis of the various binding sites on the HBV enhancer implicates several factors in mediating maximal enhancer function (Patel, unpublished results). In the case of footprint FP-I, its importance in enhancer function was tested (i) by disruption of the FP-I sequences by linker insertion mutagenesis of the *Bal*I site (nt 1225) of the enhancer fragment (*Bal*I cleavage dissects the FP-I) and (ii) by an expression vector in which a *Hpa*I-*Bal*I fragment of the enhancer was cloned in front of the chloramphenicol acetyltransferase gene. Both of these constructs produced wild-type levels of enhancer function. Taken together, these results suggest that FP-I may not be important for enhancer activity. However, since the X promoter is juxtaposed to the enhancer (38) and since factors binding to enhancers have been shown to regulate or cooperate with factor(s) on adjacent promoters (9, 20, 31), it is possible that some of the HBV enhancer binding factors (including the factor for FP-I) are also required for regulation of both the enhancer and HBV promoter elements. Its functional role in X promoter activity is presently being examined.

To date, we have identified regions within the HBV enhancer that are target sites for factors which are both ubiquitious and liver specific in nature. Recent evidence suggests that the formation of cell-specific transcription complexes requires a combinatorial synergism between both cell-type-specific and general transcription factors bound to the regulatory regions (20). The large number of HBV enhancer binding proteins and the presence of most of them in both liver and nonliver cells suggests that the action of the HBV enhancer may also involve complex interactions between specific and general transcription factors as well. Accumulation of so many target sites in the enhancer might have evolved to enable the enhancer to regulate transcription of HBV genes at various stages of clinical expression of viral infection. Similarly, such an enhancer would have gained the flexibility to be transcriptionally active at all times at various stages of liver differentiation as well. It remains to be seen whether all or some of the factors involved in binding to the HBV enhancer are required for eliciting enhancer function during the various stages of liver differentiation.

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ADDENDUM

Recently, we have been able to detect FP-VIII with HeLa nuclear extracts as well. However, this protection is at least 1 order of magnitude weaker than that seen with RLNE.

ADDENDUM IN PROOF

NF-1 purified from HeLa cells binds to the enhancer at three sites, including FP-I (1). However, with RLNE, an NF-1 oligomer does not seem to compete with the FP-I, suggesting that a factor(s) other than NF-1 might interact with that sequence.

LITERATURE CITED

1. **Ben-Levy, R., O. Faktor, I. Berger, and Y. Shaul.** 1989. Cellular factors that interact with the hepatitis B virus enhancer. Mol. Cell. Biol. **9**:1804–1809.
2. **Bulla, G., and A. Siddiqui.** 1988. The hepatitis B virus enhancer modulates transcription of the hepatitis B virus surface antigen gene from an internal location. J. Virol. **62**:1437–1441.
3. **Cereghini, S., M. Raymondjean, A. G. Carranca, P. Herbomel, and M. Yaniv.** 1987. Factors involved in control of tissue-specific expression of albumin gene. Cell **50**:627–638.
4. **Clark, L., R. M. Pollock, and R. T. Hay.** 1988. Identification and purification of EBPI: a HeLa cell protein that binds to a region overlapping the 'core' of the SV40 enhancer. Genes Dev. **2**:991–1002.
5. **Courtois, G., S. Morgan, L. Campbell, G. Fourel, and G. R. Crabtree.** 1987. Interaction of a liver-specific nucleus factor with fibrinogen and a1 antitrypsin promoters. Science **238**: 688–692.
6. **deWet, J., K. Wood, M. Deluca, D. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–757.
7. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian cells. Nucleic Acids Res. **11**:1475–1489.
8. **Farza, H., M. Madchouel, J. Scotto, P. Tiollais, C. Babinet, and C. Pourcel.** 1988. Replication and gene expression of HBV in a transgenic mouse that contains the complete viral genome. J. Virol. **62**:4144–4152.
9. **Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon.** 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. Cell **54**:943–953.
10. **Galas, D., and A. Schmitz.** 1978. DNase footprinting: a simple method for detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
11. **Ganem, D., and H. Varmus.** 1987. The molecular biology of hepatitis B virus. Annu. Rev. Biochem. **56**:651–693.
12. **Gluzman, Y., and T. Shenk (ed.).** 1983. Current communications in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes that express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
14. **Gorman, C. M., R. Padmanabhan, and B. H. Howard.** 1983. High efficiency DNA-mediated transformation of primate cells. Science **221**:551–553.
15. **Gronostajski, R. M.** 1986. Analysis of nuclear factor I binding to DNA using degenerate oligonucleotides. Nucleic Acids Res. **14**:9117–9132.
16. **Jameel, S., and A. Siddiqui.** 1986. The human hepatitis B virus enhancer requires *trans*-acting cellular factor(s) for activity. Mol. Cell. Biol. **6**:710–715.
17. **Jameel, S., and A. Siddiqui.** 1987. The HBV enhancer and trans-acting factors. UCLA Symp. Hepadna viruses **70**:65–76.
18. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
19. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelley, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
20. **Jones, N., P. Rigby, and E. Ziff.** 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
21. **Karpen, S., R. Banerjee, A. Zelent, P. Price, and G. Acs.** 1988. Identification of protein-binding sites in the hepatitis B virus enhancer and core promoter domains. Mol. Cell. Biol. **8**: 5159–5165.
22. **Landschulz, W., P. Johnson, E. Adashi, B. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2**:786–800.
23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
24. **Nagata, K., R. A. Guggenheimer, and J. Hurwitz.** 1983. Specific binding of a cellular DNA-binding protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA **80**:6177–6181.
25. **Nakahyashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. **42**:3858–3863.
26. **Ostapchuk, P., J. F. X. Diffley, J. T. Bruder, B. Stillman, A. J. Levine, and P. Hearing.** 1986. Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA **83**:8550–8554.
27. **Paonessa, G., F. Gounari, R. Frank, and R. Cortese.** 1988. Purification of a NF-1-like DNA binding protein from rat liver and cloning of the corresponding cDNA. EMBO J. **7**:3115–3123.
28. **Ptashne, M.** 1986. Gene regulation by proteins acting nearby and at a distance. Nature (London) **322**:697–701.
29. **Robinson, W. S., P. Marion, M. Feitelson, and A. Siddiqui.** 1981. The hepa-DNA-virus group: hepatitis B and related viruses, p. 57–68. *In* W. Szmuness, J. Alter, and J. Maynard (ed.), Viral hepatitis. Franklin Institute, Philadelphia.
30. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of NF-1 by DNA recognition site affinity chromatography. J. Biol. Chem. **261**:1398–1408.
31. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CCAAT-box binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334**:218–224.
32. **Sassone-Corsi, P., and E. Borrelli.** 1986. Transcriptional regulation by trans-acting factors. Trends Genet. **2**:215–219.
33. **Sassone-Corsi, P., J. Daugherty, B. Wasylyk, and P. Chambon.** 1984. Stimulation of in vitro transcription from heterologous promoters by the SV40 enhancer. Proc. Natl. Acad. Sci. USA **81**:308–312.
34. **Schlokat, U., D. Bohmann, H. Schöler, and P. Gruss.** 1986. Nuclear factors binding sequences within the immunoglobulin enhancer interact differentially with other enhancer elements. EMBO J. **5**:3251–3258.
35. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.

36. **Shaul, Y., and R. Ben-Levy.** 1987. Multiple nuclear proteins in liver cells are bound to hepatitis B virus enhancer element and its upstream sequences. EMBO J. **6**:1913–1920.
37. **Shaul, Y., W. J. Rutter, and O. Laub.** 1985. A human hepatitis B viral enhancer element. EMBO J. **4**:427–430.
38. **Siddiqui, A., S. Jameel, and J. Mapoles.** 1987. Expression of the hepatitis B X gene in mammalian cells. Proc. Natl. Acad. Sci. USA **84**:2513–2517.
39. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–158.
40. **Tagnoni, A., R. Cattaneo, E. Serfling, and W. Schaffner.** 1985. A novel expression selection approach allows precise mapping of the hepatitis B virus enhancer. Nucleic Acids Res. **13**:7457–7472.
41. **Wildeman, A., M. Zenke, C. Schatz, M. Winterzith, T. Grundstrom, H. Matthes, K. Takahashi, and P. Chambon.** 1986. Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. **6**:2098–2105.
42. **Woodworth, C., and H. Isom.** 1987. Regulation of albumin gene expression in a series of rat hepatocyte cell lines immortalized by simian virus 40 and maintained in chemically defined medium. Mol. Cell. Biol. **7**:3740–3748.