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Cellular Factors That Interact with the Hepatitis B Virus Enhancer

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An 83-base-pair-long hepatitis B virus DNA fragment efficiently activates the transcription of the heterologous globin gene promoter. This fragment contains binding sites for at least four distinct cellular factors termed E, TGT3, EP, and NF-I. E is a positively acting factor, responsive to phorbol ester. EP is apparently identical to the factor EF-C that binds to the polyomavirus enhancer. The conservation of the binding site sequences for most of these factors in the genomes of other members of the hepadnavirus family suggests that these viruses share common enhancer elements.

Human hepatitis B virus (HBV) is characterized by the mode of its replication which, unlike those of the other known DNA viruses, operates through reverse transcription of a pregenome RNA (7). The synthesis of pregenomic transcripts is therefore the crucial step in HBV replication. The elucidation of the regulation of HBV transcription is therefore of importance in understanding viral expression and replication. The first step along this line was the identification of an enhancer in the viral genome which regulates the expression of apparently all the viral promoters (6, 20, 21). To map the HBV enhancer in greater detail than done previously (20, 22), we have used a globin-based plasmid and assayed the enhancer activity by measuring the amount of globin mRNAs using the RNase A/T_1 protection technique (12). An HBV DNA fragment (nucleotides 1043 to 1266), containing the enhancer, activated the β-globin promoter in the transfected Alexander and HEp-3B cell lines (Fig. 1, fragment L). A shorter HBV fragment (fragment M) still exhibited enhancer activity, though to a lower extent. The level of enhancer activity was only slightly lower when a smaller fragment (fragment S) was inserted either at the 5′ or at the 3′ end of the β-globin gene (Fig. 1). The S fragment contains the binding site of the nuclear protein previously designated by us as E (18).

We have found that a single synthetic E site did not activate transcription (data not shown), suggesting that additional elements and *trans*-acting factors are required. In search of such factors, we fractionated nuclear extracts on a cation-exchange Bio-Rex 70 column (17). The fractions were analyzed by DNase I footprinting, using an end-labeled HBV probe that bears the sequence of the enhancer. At least three regions were protected by the 450 mM NaCl fraction; these were designated a, b, and EP (Fig. 2). An additional footprint, due to protection of the sequence TGTTT, was obtained in the 500 mM NaCl fraction and was termed accordingly TGT3. For reasons that are not clear to us the E footprint is missing in these fractionated extracts. Note, however, that the E and the EP sites are the only protected regions that were revealed when crude nuclear extracts were used (Fig. 2B). Since the TGT3 footprint overlaps with the lower portion of the E site, it is very likely that the E region is composed of two distinct recognition sequences. A simple way to test this possibility is to introduce a specific mutation at the recognition sequence of one of the factors. When the sequence CGCA, located at the center of the E site, was mutated to TTGT, the E footprint was no longer seen but the TGT3 site was now protected by the crude extract from DNase digestion (Fig. 2B), clearly demonstrating that two distinct proteins can occupy the E region. However, we do not know yet whether the two proteins bind simultaneously or if their binding is mutually exclusive.

We had noticed previously that a portion of the E region is similar to the binding site of AP-1 (1, 11, 18). To examine the possibility that AP-1 binds to the HBV enhancer in general and to the E region in particular, we performed a series of competition experiments and gel retardation assays. Addition of up to 100-fold excess of DNA containing the AP-1 binding site competed slightly for the binding of the E factor, whereas the AP-2 DNA did not compete at all (data not shown). This observation suggests that AP-1 is related but not identical to E.

To test whether E is a positive factor, the experimental strategy developed by Veldman et al. (24) was used. Oligonucleotides bearing the E-binding site were tandemly cloned as a multimer into the enhancerless plasmid $p\beta e^-$ (Fig. 3). Six copies of the E site acted as a strong enhancer element and stimulated the synthesis of β-globin RNA up to 50-fold (data not shown). Recently it was reported that AP-1 can efficiently activate a nearby promoter following 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) treatment (1, 11). We took advantage of this feature to address the question of similarity between E and AP-1. The plasmid that contains six tandem copies of the synthetic E site was introduced together with a reference plasmid into Alexander cells, which were then treated with TPA (Fig. 3). As a positive control, we used a plasmid that contains five tandem repeats of a synthetic simian virus 40 AP-1 site at the 3′ end of the β-globin gene in the $p\beta e^-$ vector. As expected, the ability of AP-1 to activate a nearby promoter was highly TPA dependent (7.5-fold induction). The HBV E site efficiently activated the β-globin promoter in the absence of TPA (compare lanes 3 and 5 of Fig. 3C), but this activation was enhanced threefold by TPA treatment (Fig. 3C). Thus, in full agreement with the gel retardation analysis, the E factor that binds to the HBV enhancer element is similar but apparently not identical to AP-1.

The analysis of the sequence of the EP-binding site revealed that it contains a 6-base-pair (bp) inverted repeat separated by 3 bp. To test the capability of this sequence to bind a nuclear protein(s), we prepared a synthetic DNA containing the EP site, incubated it with nuclear proteins, and analyzed the complex on an acrylamide gel. At least two kinds of complexes were generated (Fig. 4). To determine

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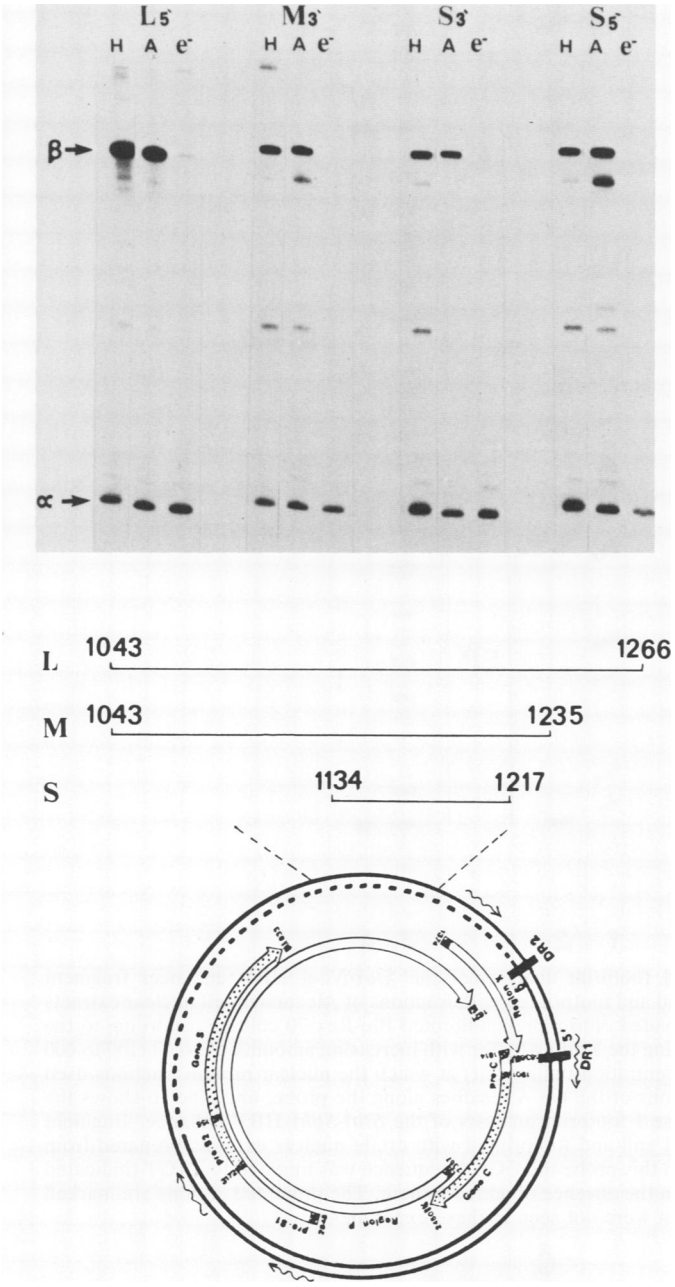


FIG. 1. Fine mapping of the HBV enhancer element. Three HBV fragments labeled L, M, and S were inserted at the 5′ or 3′ end of a β-globin reporter gene as indicated at the top of the lanes. HEp-3B cells (H) and Alexander cells (A) were transfected, by the calcium phosphate precipitation technique, with each plasmid together with a reference plasmid that containing the α-globin gene (15, 23). The fragments protected from RNase A/T_1 digestion (12) are denoted by α and β, respectively. Alexander cells were also transfected with an enhancerless β-globin plasmid (lane e^-). The bottom part of the figure displays the HBV map and the positions of the viral promoters (wavy lines).

the specificity of these complexes, we performed a series of competition experiments. Formation of the complexes was completely inhibited by the addition of excess cold synthetic EP site DNA, but not by the addition of other cold synthetic factor-binding sites (E and NF-I; Fig. 4, lanes 6 through 11). The E-binding site contains a 4-bp inverted repeat which is identical to that of the EP site except that the repeat is separated by 4 bp instead of 3 bp (Fig. 4, bottom). The lack of competition between the E- and EP-binding sites further emphasizes the specificity of the EP protein. We also noted a similarity between the EP-binding site and a sequence in the polyomavirus enhancer which binds EF-C (16) (Fig. 4, bottom). To examine whether these two factors are similar, we performed competition experiments and found that polyomavirus DNA efficiently competed for EP (Fig. 4, lanes 12 through 14), suggesting strongly that the same protein binds to both enhancer elements.

Comparison of the sequences of footprints a and b (Fig. 2) revealed that both contain the consensus sequence of the nuclear factor I (NF-I)-binding site. When a nuclear extract (prepared from Wish cells) was incubated with a DNA probe containing the HBV enhancer region, end labeled at either the minus or the plus strand, the two expected footprints, a and b, were detected (Fig. 5, lanes C). The crude nuclear extract was then fractionated on a Bio-Rex 70 column, and the fractions obtained were analyzed by DNase I footprinting. The 400 mM NaCl fraction, found to contain the binding activity, was further purified by chromatography over an affinity resin containing multimers of a synthetic NF-I-binding site. Footprint analysis showed that the purified NF-I obtained by this procedure binds to both the a and b regions (Fig. 5, lanes P).

Figure 6 summarizes the data obtained from DNase I footprinting. With the exception of duck HBV, the sequences of the binding sites for the E, TGT3, and EP factors are fully conserved among members of the hepadnavirus family (Fig. 6B), strongly suggesting that binding of these factors is required for the activity of the enhancer element. Additional evidence that the EP factor is an enhancer activator derives from the fact that this factor, or a highly related one, binds to the polyomavirus enhancer (16). Based on a computer search, we have also learned that a similar binding site is located in the long terminal repeat region of the equine infectious anemia virus (4). Therefore, we believe that the EP protein is a *trans*-activator of a number of viral enhancers. The observation that this factor is ubiquitous and is found in a variety of cell lines (16; unpublished data) further supports this hypothesis.

On the basis of recognition sequence similarity, competition experiments, and functional studies of the E protein, we conclude that the E protein is related to the recently defined transcriptional factor AP-1 (1, 11). This factor belongs to a family of proteins which share a common DNA-binding domain that is highly similar to that of the yeast transcriptional factor GCN4 (2, 21). Six copies of a 26-bp oligomer bearing the sequence of the E site efficiently enhanced the activity of the β-globin promoter. The activity was further enhanced by TPA, a behavior characteristic of AP-1 family proteins (1, 11). The exact mechanism responsible for the TPA effect remains to be elucidated. However, TPA is known to exert its biological effects via the activation of protein kinase C (14), and it is therefore speculated that site-specific phosphorylation of these proteins is responsible for their increased activity (1).

NF-I was originally identified as a cellular factor which is required for in vitro replication of adenovirus (13). NF-I-binding sites were also identified in the promoter regions of a number of cellular and viral genes. The first indication that NF-I is required for the efficient activation of a promoter came from the analysis of the HBV S gene promoter (19). In this case, deletions that removed a portion of or the whole NF-I-binding site resulted in a 5- to 10-fold reduction in

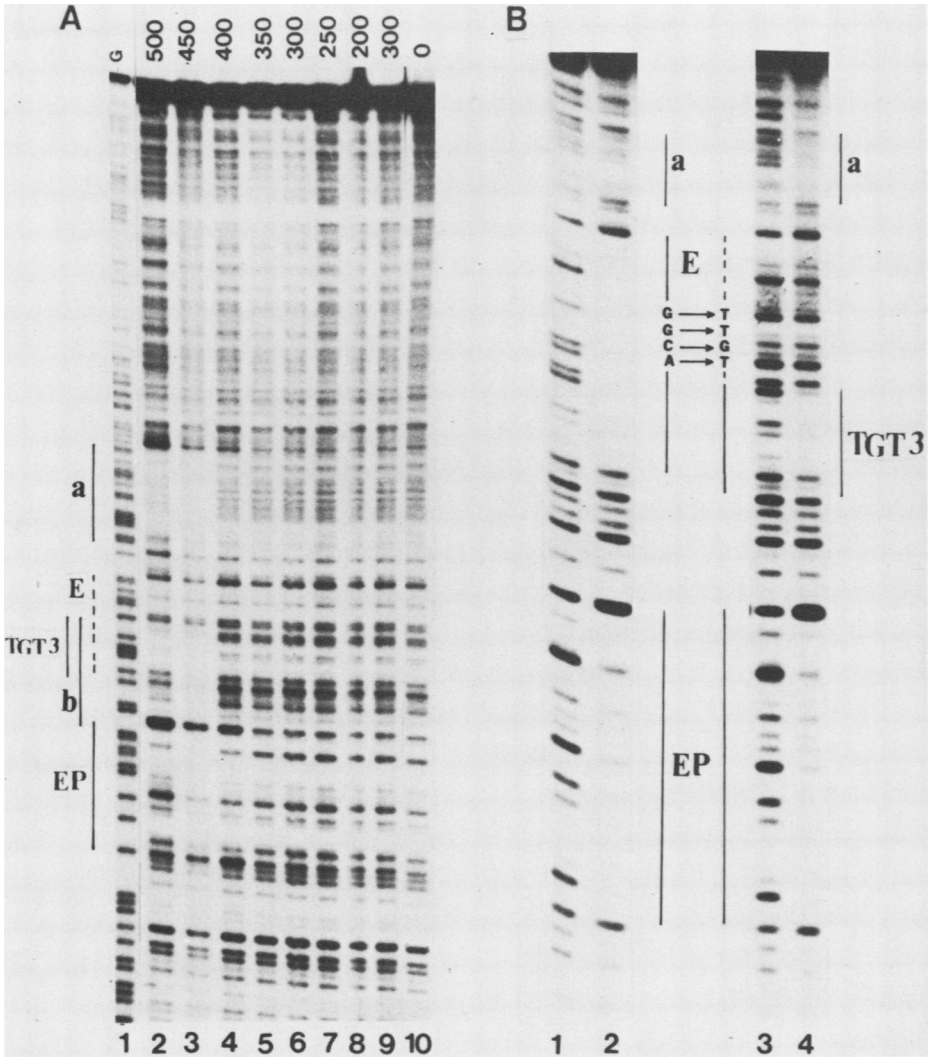
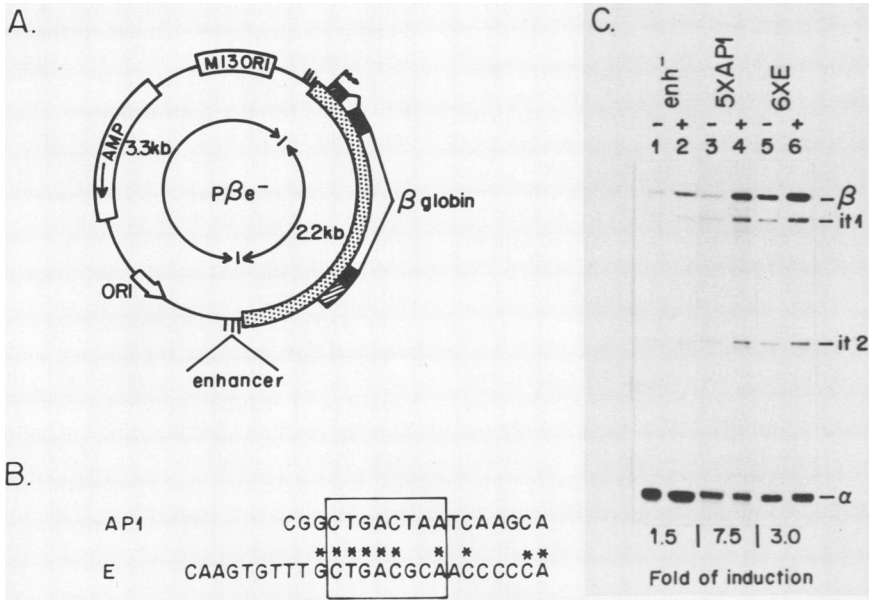


FIG. 2. DNase I footprint analysis of HBV enhancer. (A) DNase I footprint analysis of the *Stu*I-*Nco*I HBV enhancer fragment (nucleotides 1115 to 1375). The fragment was ^{32}P end labeled at the *Stu*I end and footprinted with fractions of Alexander cell nuclear extracts obtained by chromatography on Bio-Rex 70. Nuclear extracts (5) were loaded onto a preequilibrated Bio-Rex 70 column according to the published protocol (17). The DNA-binding activity was eluted stepwise using the loading buffer with increasing amounts of NaCl (100 to 600 mM at intervals of 50 mM). The top of each lane indicates the NaCl concentration (millimolar) at which the nuclear protein fractions used in the DNase protection assay were step-eluted. Lane 1 shows the positions of the G+A residues along the probe, and lane 10 shows the degradation pattern observed in the absence of added protein. (B) DNase I footprint analysis of the *Stu*I-*Sph*I HBV enhancer fragment (nucleotides 1115 to 1235). The fragment was ^{32}P end labeled at the *Stu*I end and footprinted with crude nuclear extracts prepared from Alexander cells (lane 2). For lane 3 a similar probe was used except that in this probe the GGCA sequence was mutated to TTGT (indicated by small arrows). Lanes 1 and 3 show the degradation patterns observed in the absence of added protein. The protected regions are marked by vertical lines. Broken lines indicate the expected protected regions that were not seen in these experiments.



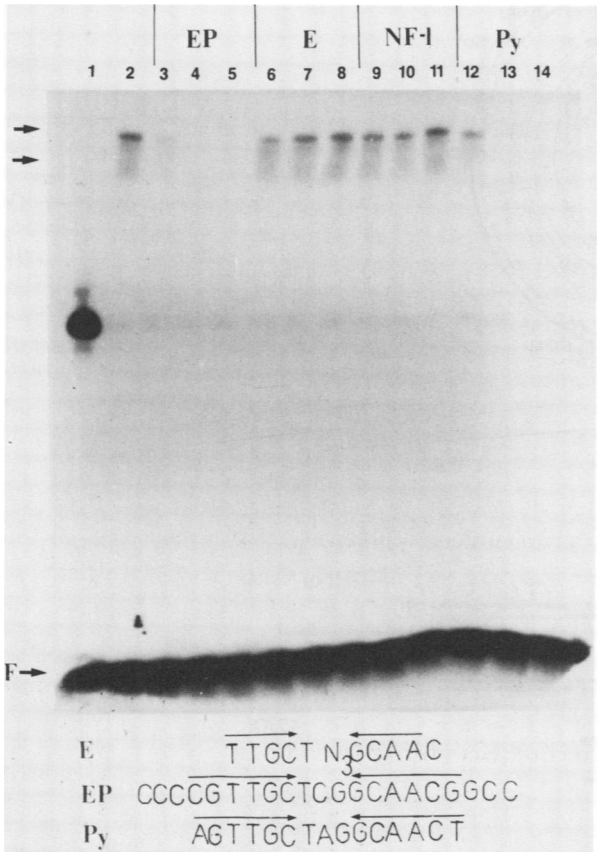


FIG. 4. Analysis of EP by gel retardation assay. Double-stranded, ^{32}P-end-labeled, synthetic EP-binding site (sequence is shown at the bottom) was used as a probe. Complexes formed between the probe and Alexander cell nuclear extracts in the absence (lane 1) or presence (lanes 2 through 14) of poly(dI · dC) as a nonspecific competitor were resolved by gel electrophoresis (3). The competitor DNAs included in the reactions are indicated on top. The following amounts of competitor DNA were used: 1 ng (lanes 3, 6, 9, and 12), 10 ng (lanes 4, 7, 10, and 13), or 100 ng (lanes 5, 8, 11, and 14). The EP, E, and NF-I DNAs used as competitors are identical to those used for Fig. 3. As a polyomavirus enhancer (Py) we used the *Pvu*II fragment that contains the viral C enhancer element (16). At the bottom of the figure the sequences of E, EP, and PY are compared, with arrows indicating the invertedly repeated sequence motifs. The thick arrows indicate the two complexes generated consistently in a number of independent experiments. F, Free probe.

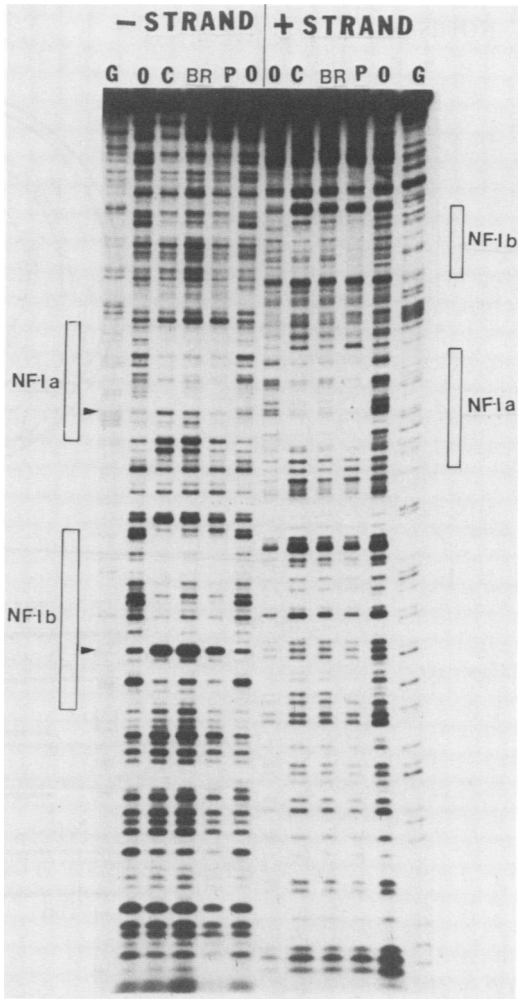


FIG. 5. Footprint analysis of the HBV enhancer element and purified NF-I. HBV DNA fragment (*Stu*I-*Nco*I), labeled either at the *Stu*I end (−STRAND) or at the *Nco*I end (+STRAND), was incubated with the following extracts: crude nuclear extracts prepared from Wish cells (lanes C), 300 to 400 mM NaCl elution fractions from a Bio-Rex 70 column (lanes BR), and Bio-Rex 70 fractions that were further purified on an affinity column containing the NF-I recognition sequence as a ligand (lanes P). Lanes O, No extract was included; lanes G, products of chemical cleavage at G residues. Arrowheads identify DNase I-hypersensitive sites. The Bio-Rex 70 chromatography and affinity purification were done as described for Fig. 2. For affinity purification of NF-I, Bio-Rex 70 fractions shown to be positive for NF-I were pooled and applied to DNA-affinity columns prepared with a sequence from an NF-I-binding site at the HBV S promoter (19), 5′-GGACGACTGGC CAGCAGCCAACCAA-3′ (HBV position 3005 to 3027). The affinity resin was prepared as described by Kadonaga and Tjian (10). Affinity purification was performed essentially as described by Lee et al. (11), except that a single cycle of purification was performed. We estimated that we obtained about 10,000-fold-enriched NF-I.

FIG. 3. Multiple copies of the AP-I-binding site and the E element can enhance TPA-inducible transcription. (A) Map of plasmid pβe$^-$ described previously by Ondek et al. (15). (B) Comparison of the nucleotide sequences of the AP-I and E sites that were inserted into the 3′ end of pβe$^-$. Homolog bases are indicated by stars, and the sequence reported to be essential for AP-I binding is boxed. (C) Analysis of α- and β-globin RNA probe protected from RNase digestion by cytoplasmic RNA isolated from transfected Alexander cells, either treated (+) or not treated (−) with TPA. For treatment with TPA, cells first received 0.5% fetal calf serum, and then TPA at a concentration of 100 ng/ml was added 14 h before cells were harvested. The correctly initiated reference α-globin (132 nucleotides) and tested β-globin (350 nucleotides) protected fragments are marked α and β, respectively. The incorrectly initiated β-globin transcripts are marked it 1 and it 2, following the terminology used by Ondek et al. (15). The following plasmids were used: pβe$^-$ (lanes 1 and 2); 5XAPI, a plasmid with five copies of an AP-I binding site (a generous gift of M. Karin) (lanes 3 and 4); 6xE, a plasmid that contains six copies of a 25-mer E site at the 3′ end of the globin gene (lanes 5 and 6). The ratio between TPA treated (+) and untreated (−) is shown at the bottom of the lanes as the fold induction obtained by TPA. The numbers are the average of three independent experiments.

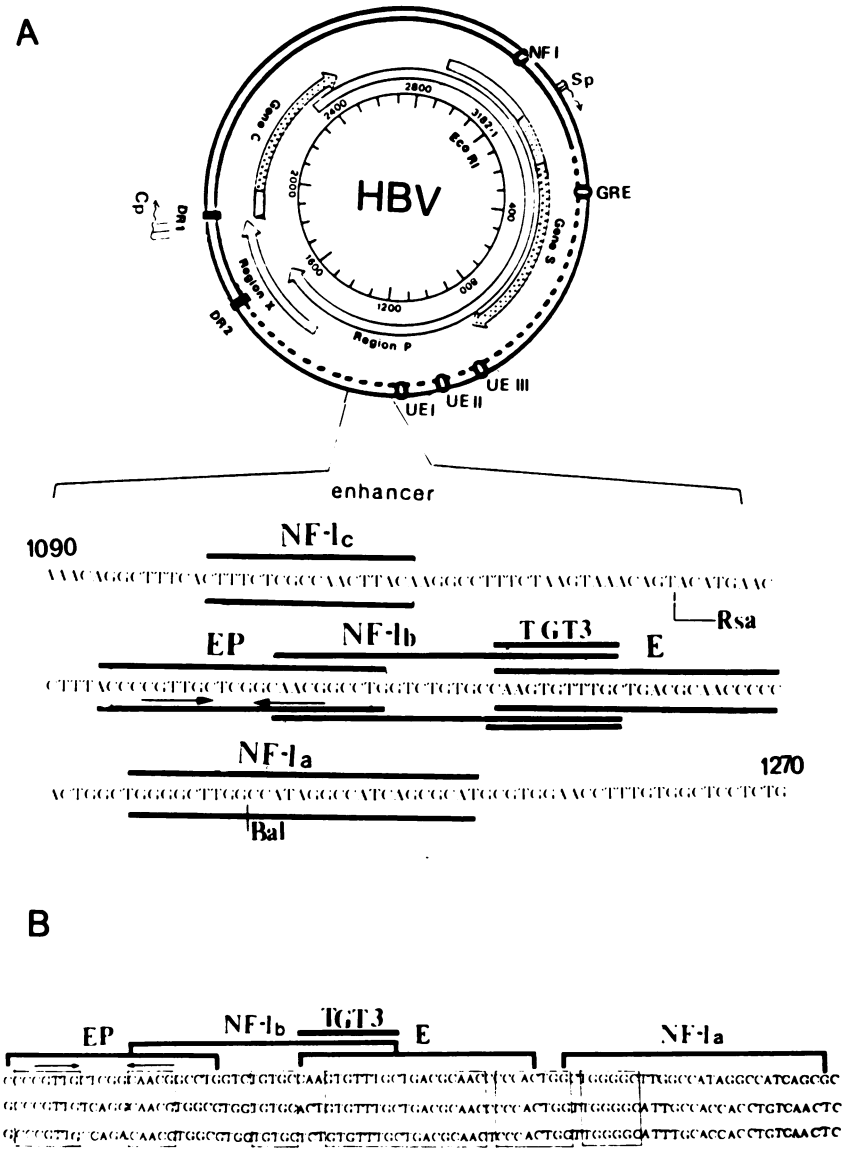


FIG. 6. Map of protein-binding sites of the HBV enhancer element and its conserved sequences among the members of hepadnavirus family. (A) HBV map and diagrammatic summary of the HBV enhancer sequences protected by nuclear extracts. The HBV map shows the relative position of the major viral genes, open reading frames (P and X), and viral direct repeats (DR1 and DR2). The positions of the major viral promoters (CP and SP) are indicated by wavy arrows. Also shown are the previously published binding sites for the following nuclear factors: NF-I (19); GRE (unpublished data); and UE1, UE2, and UE3 (18). The sequence of the enhancer region from positions 1090 to 1270 (double thick lines to indicate the protected regions) is shown. The *Rsa*I-*Bal*I sites used to generate the 83-bp probe in Fig. 1 are also indicated, as is the position of an additional NF-I site designated NF-1c that is not described in this paper. (B) Comparison of the sequence of the HBV enhancer element with homologous sequences found in woodchuck HBV (WHBV) and ground squirrel HBV (GSHV). Stretches of four bases or more that are conserved among the viruses are boxed. The positions of factor-binding sites are labeled by thick lines.

transcription. More recent experiments suggest that NF-I and the CCAAT-binding transcription factor (CTF) are identical or highly related proteins (9). Since during the course of purification of NF-I/CTF several other proteins with distinct molecular weights were copurified, it is likely that there exists a set of proteins with a common DNA-binding domain which recognizes very similar DNA sequence motifs.

What are the essential components of the HBV enhancer? To identify the subcomponents of this element, 20- to 26-bp-long synthetic DNA fragments that contain the recognition sequences of the above factors were tested for enhancer function. Neither of them alone exhibited enhancer activity (data not shown), a fact that suggests strongly that the activation of transcription is obtained by combinatorial mechanisms. The rather low conservation of the NF-I-binding sites within the enhancers of different members of the hepadnavirus family (Fig. 6) suggests that NF-I per se is perhaps not essential for enhancer activity and may be replaced by another cellular factor.

The HBV enhancer was shown to be active mostly in liver cells, raising the possibility that this element carries the recognition sequence for a liver-specific *trans*-acting factor(s). The four distinct cellular factors that interact with the 83-bp HBV fragment seem to be ubiquitous in that they are present in a large number of cell lines (unpublished data). We have previously reported that the sequence motif TGTT

TGCT is found in a number of liver-specific promoters (18), and more recently it was also found in enhancers (8). Although the role of this sequence motif is not known, it may play an essential part in the determination of liver-specific expression. The present observation that a cellular factor indeed recognizes this element (the TGT3 factor) further supports our hypothesis. Further analysis of this motif and its mutant derivatives, and of the TGT3 factor, will be required to establish its role in the determination of liver-specific gene expression.

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