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Hierarchic and Cooperative Binding of the Rat Liver Nuclear Protein C/EBP at the Hepatitis B Virus Enhancer

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We used the enhancer-binding protein C/EBP as a model to study the nature and the complexity of interaction of an enhancer-binding protein with its target DNA. We found that bacterially expressed C/EBP binds the hepatitis B virus enhancer at multiple sites in a hierarchic and cooperative manner. At low concentrations, only the E element is occupied, but at higher concentrations, additional sites are filled including a site that binds EP, a crucial enhancer-activating protein. This pattern of C/EBP binding may explain the concentration-dependent effect of C/EBP on enhancer activity.

The hepatitis B virus (HBV) enhancer is active in a liver cell environment and strictly tissue specific in conjunction with the promoter of the viral core gene (8, 9, 14). This enhancer interacts with a number of cellular DNA-binding proteins, most of which seem not to be liver specific (1). However, a heat-stable protein found in hepatoma nuclei has been shown to interact at two regions of the HBV enhancer, E and UEI (12). Recently, a heat-stable protein that binds at two regions of the HBV enhancer similar to or very close to the E and UEI sites was isolated from rat liver nuclei (10, 11). This protein, termed C/EBP, found in tissues in which the metabolism of lipids is an important part of their normal physiology, interacts with a large number of viral enhancers and promoters (2, 10, 11). C/EBP activates the promoter of liver- and adipocyte-specific genes (3, 6). Thus, it is very likely that C/EBP or a highly related protein plays an important role in activation of the HBV enhancer in liver cells. The availability of bacterially expressed C/EBP provides us with a tool that permits the study of the mode of interaction of this protein with the HBV enhancer. To investigate the binding of C/EBP at the E element and to define the sequence requirement of this interaction, we prepared a number of synthetic probes of 26-base-pair DNAs that contain either the sequence of the E element or its derivative mutants. These probes were incubated with the bacterially expressed fusion protein β-galactosidase (β-Gal)-C/EBP (11), and the complexes were resolved on a native polyacrylamide gel (Fig. 1). The E element bound this protein efficiently (complex A), and the specificity of this complex was determined by competition experiments using homologous DNA as a competitor. This binding was very sequence specific and was abolished upon introduction of a specific mutation (Fig. 1B, mutant Em-5). We infer from these results that the E element binds C/EBP. A similar

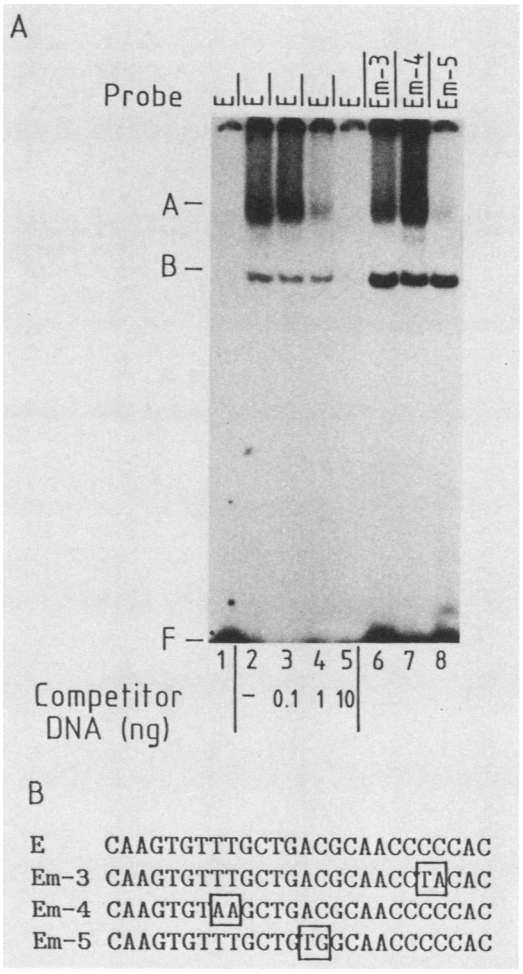

FIG. 1. HBV enhancer E element binds C/EBP, as analyzed by gel retardation. Double-stranded synthetic oligonucleotide sequences (panel B) were ^{32}P labeled, 0.2 ng (20,000 cpm) of the probe was incubated with β-Gal-C/EBP fusion protein prepared from bacteria that were infected with L-20 phage (kindly provided by S. McKnight) (11), and the complexes were resolved on a native polyacrylamide gel (15) (panel A). Lane 1, Migration of free probe (F) without addition of extracts; lane 2, no E element; lanes 3 to 5, increasing amounts of cold oligonucleotide E (indicated at the bottom of the lanes) added as a competitor; lanes 6 to 8, mutants Em-3, Em-4, and Em-5, respectively. Band A formed by specific interaction of the probe with the fusion protein. Band B is a nonspecific complex. In panel B, the modified sequences are boxed.

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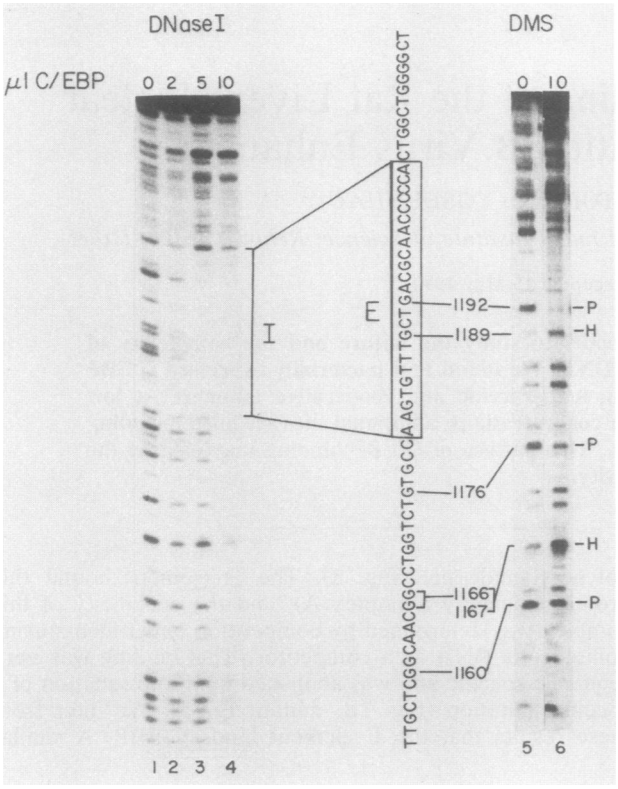
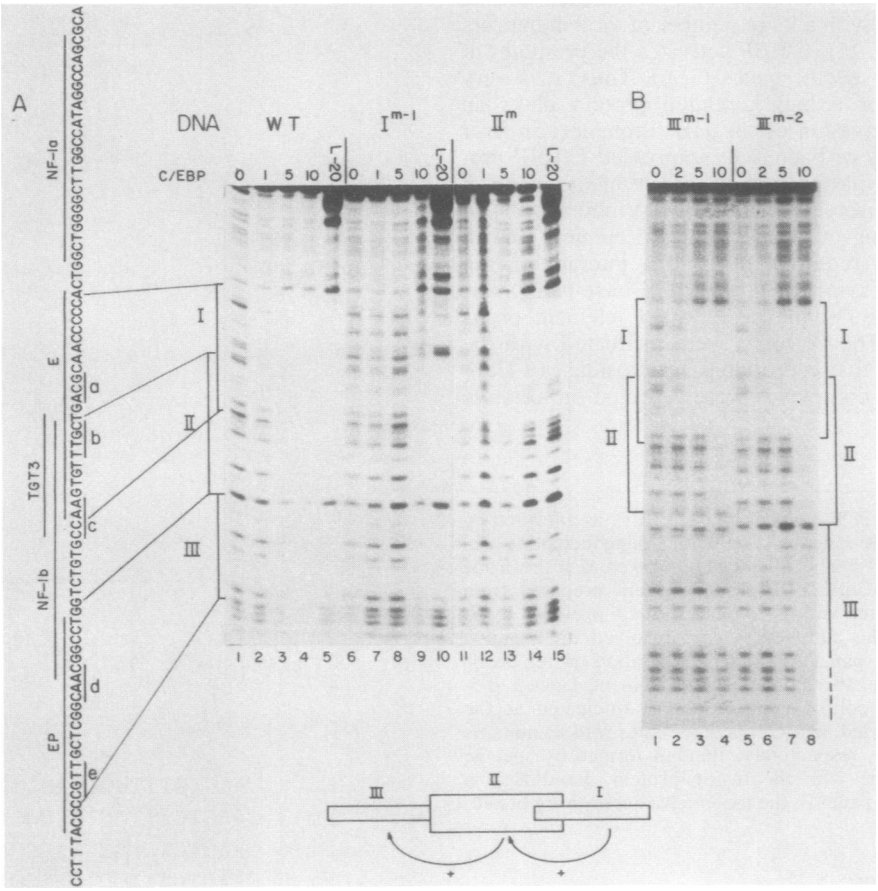


FIG. 2. C/EBP binds at multiple sites on the HBV enhancer. The *Stu*I-*Sph*I (nucleotides 1115 to 1235) HBV DNA fragment that contains the enhancer sequence (14) was ^{32}P end labeled at the *Stu*I site and used for DNase I (7) and DMS protection (11). The protein used was purified native C/EBP (5 ng per μl) that was synthesized in bacteria (kindly provided by S. McKnight and W. Landschulz) (12). The sequence of protected region I exactly matches that of the E region reported previously (14). In the DMS protection experiment, the fully or partially protected G residues as determined by densitometry scanning were labeled with P and the hypercleared G residues were labeled with H. The exact positions of these G residues are shown on the HBV sequence.

conclusion was derived from DNase I footprinting and DMS protection experiments (Fig. 2). In these experiments, about 1 μl of the protein was sufficient for full protection of the E region (Fig. 2, region I). This region is almost identical to the protected region 4 mapped previously by Landschulz et al. (11) on the HBV enhancer. However, upon addition of increasing amounts of C/EBP to the binding reaction mixture, the protected region was extended and occupied most of the enhancer sequence (Fig. 2, lanes 4 and 6). These secondary binding sites are designated II and III, and they overlap with the binding sites of the NF-1b and EP proteins that were defined previously (1). This hierarchic binding of C/EBP raises the possibility of cooperative interaction of the C/EBP that binds to site I with those that bind to sites II and III. To address this possibility, each of these sites was mutated and the capacity of the generated mutants to bind



C/EBP was determined. A mutation at site I (I^{m-1}) not only abolished the binding of C/EBP at this site but also reduced the affinity of sites II and III by about fivefold (Fig. 3, lanes 3 and 8). This suggests cooperative binding of C/EBP at sites I and II plus III. Next, site II was mutated (II^m) and analyzed. In the concentration range of C/EBP used by us, this mutant bound C/EBP only at site I (Fig. 3A, lanes 12 to 14). Thus, the occupation of site II is a prerequisite for that of site III, a behavior which is compatible with the cooperative nature of the binding of C/EBP at these sites. Two additional mutants were constructed; each contains a mutation at site III, and they are designated III^{m-1} and III^{m-2}. Only III^{m-1} lost the capacity to bind C/EBP at site III (Fig. 3B, lanes 4 to 8). However, in that case the binding at sites I and II was not changed, as deduced from the fact that the concentration of C/EBP that was required to fill these sites was equal in both mutants (Fig. 3B, lanes 2 and 6). This suggests that the mode of cooperation within the C/EBP proteins is unidirectional, a conclusion which is compatible with the notion of hierarchic binding of C/EBP at the HBV enhancer.

Recently, the portion of the C/EBP polypeptide that is required for direct interaction with DNA has been identified and localized at its carboxyl terminus (11, 12). To address the question of whether this portion of C/EBP is sufficient to show cooperative binding activity, we used the L-20 clone (11). In this clone, about 18 kilodaltons of the carboxyl terminus of C/EBP is expressed as a β-Gal-C/EBP fusion protein of about 140 kilodaltons (11). Interestingly, this fusion protein behaves in our in vitro analysis exactly the same as the native 42-kilodalton C/EBP. It occupies all three binding sites (Fig. 3A, lane 5) and binds either only to sites II plus III once site I is mutated (lane 11) or to site I alone once site II is mutated (lane 15). We infer from these results that the information required for cooperative binding activity of C/EBP resides at the carboxyl terminus of C/EBP. We also addressed the question of the effect of spacing on cooperativity (Fig. 4). Sequences between site I and II were modified either by substitution or by deletion. Both mutations affect the binding affinity of C/EBP at sites II and III. However, the effect was more dramatic in a deletion mutant in which no occupancy of these sites was observed even at the highest concentrations of C/EBP (compare lanes 4 and 8). We conclude that there is some spacing requirement for maximal occupation of the C/EBP binding sites at the HBV enhancer.

It is clear from the aforementioned experiments that C/EBP binds cooperatively at multiple sites at the HBV enhancer in a hierarchic manner. At low concentrations of C/EBP, only site I (also termed the E element) is occupied. It was previously shown that the E element is a positively acting enhancer element and that a mutant of this element (Em-5) that lost the capacity to bind C/EBP (Fig. 1) also lost enhancer activity (5). At high concentrations of C/EBP, apart from the E region, additional sites on the HBV enhancer are occupied, some of which were shown to bind distinct nuclear proteins that are essential for enhancer activity. For example, sites I^2, II, and III partially comapped

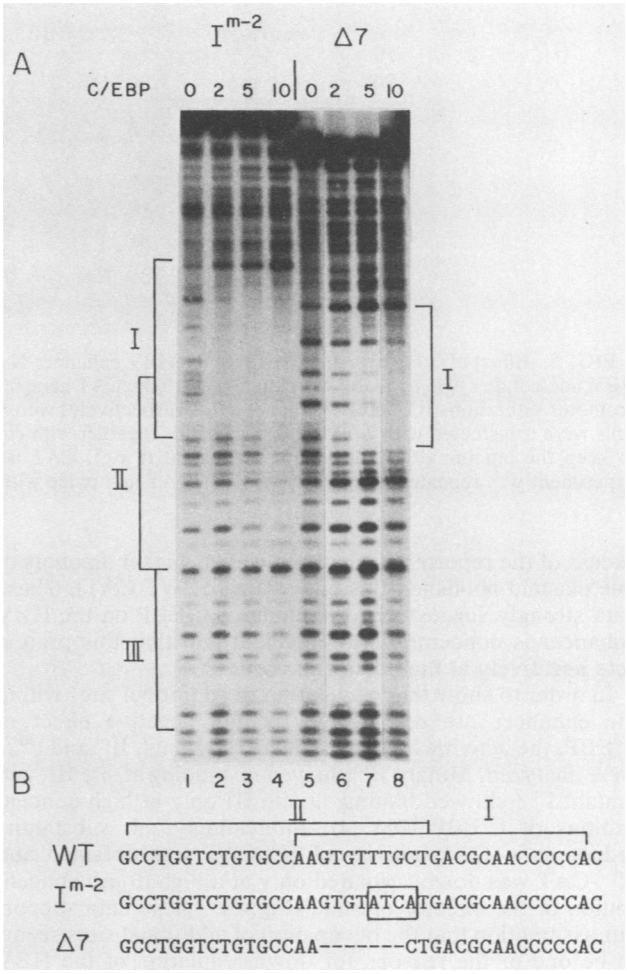


FIG. 4. Effect of spacing on C/EBP cooperation. (A) Wild-type and mutated probes were subjected to DNaseI footprinting after incubation with increasing amounts (shown in microliters at the top of lanes) of the bacterially expressed native C/EBP. (B) In I^{m-2} DNA, the sequence TTGC was modified to ATCA (boxed region), and in Δ7 DNA, 7 base pairs were deleted (broken line). WT, Wild type.

with the binding sites of the TGT3, NF-1b, and EP proteins, respectively (Fig. 3), which are essential constituents of the HBV enhancer (4). With the likely assumption that the binding of EP and C/EBP at these sites is mutually exclusive, it is very likely that at high concentrations C/EBP will repress the activity of the HBV enhancer. To investigate this possibility, HepG2 cells were transfected with a chloramphenicol acetyltransferase (CAT) reporter plasmid in which the CAT gene is under regulation of the HBV enhancer and X promoter (16) together with the MSV-C/EBP plasmid that directs the synthesis of C/EBP (6), and CAT activity was measured (Fig. 5). About a 10-fold induction in CAT activity was obtained with 0.4 μg of MSV-C/EBP (about 5 molar

FIG. 3. Cooperative binding of C/EBP at the HBV enhancer. The HBV enhancer probe described in Fig. 2 was incubated with increasing amounts (shown in microliters at the top of lanes) of bacterially expressed purified native C/EBP or β-Gal-C/EBP fusion protein (L-20) and subjected to footprinting. Mutated DNA was used as follows: I^{m-1}, the sequence ACGC (a) was modified to TGTT; II^m, the sequence CCAA (c) was modified to TTTT; III^{m-1}, the sequence CAAC (d) was changed to AGGA; and III^{m-2}, the sequence CGTT (e) was modified to AAGA. Protected regions were designated I to III and aligned onto the HBV enhancer sequence. The previously defined binding sites of several cellular factors are also shown (1). Cooperative binding of C/EBP is shown at the bottom of the figure. WT, Wild type.

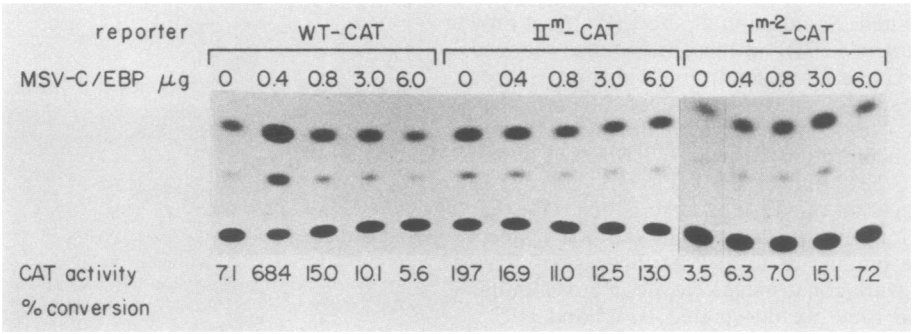


FIG. 5. Effect of C/EBP on the activity of the HBV enhancer-X promoter complex. Reporter plasmid EX-CAT (WT-CAT), which contains HBV nucleotides 1043 to 1355, was cloned next to the CAT gene in the CAT-O plasmid (6). This region contains the enhancer and the X gene promoter. Mutations II^m and I^{m-2} (Fig. 3 and 4, respectively) were also introduced to construct the II^m-CAT and I^{m-2}-CAT plasmids. HepG2 cells were transfected with 2 μg of these plasmids together with either an increasing amount of MSV-C/EBP plasmid (6) or a carrier plasmid to keep the amount of DNA per plate constant (8 μg). CAT activity was measured and quantitated as reported previously (16). This experiment was repeated four times with WT-CAT and twice with both II^m-CAT and I^{m-2}-CAT, with essentially similar results.

excess of the reporter plasmid); however, higher amounts of this plasmid abolished this effect (Fig. 5, WT-CAT). These data strongly suggest that the effect of C/EBP on the HBV enhancer is concentration dependent and that this protein acts negatively at high concentrations.

In order to show that occupation of additional sites within the enhancer are responsible for the negative effect of C/EBP, the activities of two enhancer mutants, II^m and I^{m-2}, were analyzed. Mutant II^m showed no binding at site III, and mutant I^{m-2} showed binding at site III only at high concentrations of C/EBP (Fig. 4). Interestingly, no substantial reduction in CAT activity of II^m-CAT was obtained and I^{m-2}-CAT was down regulated only at a high (6 μg) concentration of the effector plasmid (Fig. 5). These data support our assumption that the occupation of additional sites seems to be one of the reasons for down regulation of the HBV enhancer by C/EBP. However, since these enhancer mutants behave differently than the wild-type enhancer, i.e., II^m-CAT is not induced by C/EBP and I^{m-2} is activated only at a high concentration of the effector plasmid, it is very likely that additional factors are also involved in this process.

The effect of C/EBP is therefore unique, since it acts positively at low concentrations but negatively at high concentrations. A similar binding pattern is found in other viral enhancers such as those of simian virus 40 and polyomavirus. In both cases, at high concentrations, C/EBP occupies several additional regions within these enhancers (11), some of which are the binding sites of other positively acting factors. It is therefore conceivable to assume that the mode of effect of C/EBP on these enhancers is also concentration dependent.

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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. **Birkenmeir, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3**:1146–1156.
3. **Christy, R. J., V. W. Yang, J. M. Natambi, D. E. Griman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev. **3**:1323–1335.
4. **Dikstein, R., O. Faktor, R. Ben-Levy, and Y. Shaul.** 1990. Functional organization of the hepatitis B virus enhancer. Mol. Cell. Biol. **10**:3683–3689.
5. **Faktor, O., S. Budlovsky, R. Ben-Levy, and Y. Shaul.** 1990. A single element within the hepatitis B virus enhancer binds multiple proteins and responds to multiple stimuli. J. Virol. **64**:1861–1863.
6. **Friedman, A. D., W. H. Landschulz, and S. L. McKnight.** 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. **3**:1314–1322.
7. **Galas, D., and A. Schmitz.** 1987. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
8. **Honigwachs, J., O. Faktor, R. Dikstein, Y. Shaul, and O. Laub.** 1989. Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and the enhancer. J. Virol. **63**:919–924.
9. **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.
10. **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.
11. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2**:786–800.
12. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**:1681–1688.
13. **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.
14. **Shaul, Y., W. J. Rutter, and O. Laub.** 1985. A human hepatitis B viral enhancer element. EMBO J. **4**:427–430.
15. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds a conserved sequence motif in transcription control elements of immunoglobulin genes. Nature (London) **319**:154–158.
16. **Treinin, M., and O. Laub.** 1987. Identification of a promoter element located upstream from the hepatitis B virus X gene. Mol. Cell. Biol. **7**:545–548.