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Transcriptional Activation and Repression by Cellular DNA-Binding Protein C/EBP

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A putative transcription factor, C/EBP, isolated from rat liver nuclei, has been shown to bind to at least two different sequence motifs: the CCAAT promoter domain and a core sequence [GTGG(T/A)(T/A)(T/A)G] common to many viral enhancers, including simian virus 40 and human hepatitis B virus. It has been proposed that C/EBP might function as a positive transcription factor by facilitating the communication between promoter and enhancer elements through its dual binding activities to DNA. Surprisingly, results from three different approaches suggest that C/EBP functions as a transcriptional repressor to hepatitis B virus and simian virus 40. Further investigation indicated that C/EBP can function as both a transcriptional activator and a repressor, depending on the reporter gene system.

Worldwide there are 200 million chronic carriers of hepatitis B virus (HBV) (reviewed in references 3, 24, and 36). HBV is strongly associated with liver diseases such as acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Replication of HBV in tissue culture has recently been made possible by stable transfection of human hepatoma cell lines HepG2 (31, 35) and Huh-6-C15 (38) and rat Morris hepatoma cell line 7777 (33). Data generated from these studies suggest that many other hepatoma cell lines are not permissive for HBV replication. The molecular basis of the permissivity of HBV replication remains unclear. Mechanisms of permissivity may be related to tissue tropism of HBV infection.

Several liver-specific transcription factors have recently been characterized (Abstr. Meet. Regulation of Liver Gene Expression, Cold Spring Harbor, 1989). Since HBV is highly hepatotropic (3, 24, 36), it is conceivable that the pathogenesis and regulation of HBV gene expression are under the influence of liver-specific transcription factors (for a review, see reference 25). In fact, a 3.5-kilobase, HBV-specific pregenomic mRNA is believed to be the template of HBV reverse transcription (3, 24, 36).

The recent success of cloning and expressing a putative transcription factor, C/EBP, in *Escherichia coli* (19) has provided conclusive evidence that this protein factor can bind to enhancers associated with simian virus 40 (SV40), HBV, murine sarcoma virus, and polyomavirus (15). Furthermore, it can also bind with the CCAAT motif associated with the herpesvirus thymidine kinase (TK) gene and the murine sarcoma virus long terminal repeat (LTR) (8). C/EBP factor is the first protein proposed to contain a leucine zipper structure (20), which has recently been shown to be important for DNA-binding activity through protein dimerization (4, 11, 17, 21, 27, 29, 30, 39). Within a 60-amino-acid sequence, C/EBP has been found to bear a moderate degree of sequence homology to both *myc* and *fos* oncogene products (19). This protein is therefore believed to be a positive transcriptional regulator (19). Consistent with this hypothesis are results from studies of several liver-specific gene promoters including albumin (7, 12, 22), transthyretin, and α_1-antitrypsin (2).

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To elucidate issues of tissue tropism and permissivity of HBV replication, we have conducted a series of cotransfection experiments and investigated the relationship between the host liver transcription factor C/EBP and HBV gene expression. Our data provide the first evidence that C/EBP behaves as a repressor of HBV transcription.

MATERIALS AND METHODS

Plasmid constructions. All plasmids were constructed by standard DNA-cloning procedures (23). For construction of SK, a wild-type copy of the HBV genome (subtype ayw) was inserted into the *Eco*RI site of pBluescript SK+; the resulting HBV monomer was partially digested with *Eco*RI, and another HBV copy was inserted into this *Eco*RI site. A tandem HBV dimer plasmid (SK) was cloned to serve as a transcription template in HepG2 cells. For construction of plasmid 36; a point mutation was introduced into the X open reading frame of the HBV genome by site-directed mutagenesis (18). This mutation causes premature termination of X gene synthesis at amino acid 23, and will be described elsewhere (unpublished data).

For construction of R18 and R44, the chloramphenicol acetyltransferase (CAT) gene of plasmid RSV-CAT was replaced by a 1.9-Kb DNA fragment which contains the entire C/EBP coding region (19). First, the RSV-CAT plasmid was doubly digested with *Hin*dIII and *Bam*HI to remove the CAT gene; the *Hin*dIII and *Bam*HI ends of the remaining Rous sarcoma virus (RSV) LTR vector were filled in with Klenow enzyme in the presence of 0.2 mM dATP, dTTP, dCTP, and dGTP. Second, the C/EBP-encoding region plus a synthetic eucaryotic translation signal in front of its first ATG was isolated as an *Eco*RI-*Hin*dIII fragment from plasmid KS.TC/EBP (obtained from S. McKnight, Carnegie Institution of Washington); both ends of this *Eco*RI-*Hin*dIII fragment were converted into blunt ends with Klenow fragment as described above. Finally, the C/EBP fragment and RSV LTR vector (molar ratio, 1:1; total DNA concentration, 50 μg/ml) were ligated with T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Recombinants were identified by colony hybridization with GeneScreen filters (Du Pont, NEN Research Products, Boston, Mass.). The orientation of C/EBP insertion into the RSV vector was determined by diagnostic restriction enzyme digestion. As a

result, R18 has the C/EBP gene in a sense orientation with regard to RSV LTR, whereas R44 is a C/EBP antisense construct.

pSV40gpt was purchased from Pharmacia, Inc., Piscataway, N.J. The bacterial *gpt* gene is under the control of the SV40 early promoter and enhancer.

pSV232A.CAT.LRS was obtained from the laboratory of T. Kadesh, University of Pennsylvania. This plasmid contains the bacterial CAT gene which is under the control of an SV40 early promoter preceded by two copies of SV40 polyadenylation signal. Another copy of the SV40 polyadenylation signal is 3′ downstream to the CAT gene. The 180-base-pair SV40 enhancer (nucleotides 93 to 272) is located at the end (see Fig. 4a).

TK-hGH was purchased from Nichols Institute, San Juan Capistrano, Calif. In TK-hGH, the thymidine kinase (TK) promoter of herpesvirus directs the expression of the human growth hormone gene (see Fig. 4a).

pEN1 has a 193-base-pair sequence from the HBV enhancer with one of the C/EBP-binding sites cloned into the pUC19 polylinker region (a gift from C. H. Lee, Indiana University) (Fig. 1a).

All these plasmids were prepared from their bacterial host *E. coli* HB101 by the large-scale alkali protocol (23).

Cell lines and media. HepG2 cells were derived from a hepatoblastoma (1) and have been shown to be permissive for HBV replication (31, 35). Q7 cells were derived from a Morris hepatoma of rat origin. These cells have been shown to support HBV replication when stably transformed by an HBV dimer construct (33). Both cell lines were maintained in high-glucose Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah) in the presence of 5% CO_2 at 37°C.

DNA transfection. Calcium phosphate coprecipitation was used to transfect plasmids into HepG2 and Q7 cells as described previously (33). At 16 to 20 h prior to transfection, cells were trypsinized and plated at a density of approximately 5×10^5 cells per 10-cm dish. An appropriate amount of plasmids plus sheared HepG2 genomic DNA, in a total amount of 35 μg/10-cm dish, was used in each transfection.

RNA isolation. At 40 h after transfection, the cells were washed with ice-cold phosphate-buffered saline and then lysed with 4 M guanidine thiocyanate solution. The lysates were sheared with 18-gauge needles, loaded onto a 5.7 M CsCl cushion and spun at 35,000 rpm for 16 h in a Beckman SW50 rotor. The RNA pellets were dissolved in TES buffer (10 mM Tris hydrochloride [pH 7.4], 5 mM EDTA, 1% sodium dodecyl sulfate SDS), extracted twice with chloroform-butanol (80:20), and then precipitated with ethanol. The RNA pellet from microcentrifugation was dried and dissolved in diethyl pyrocarbonate-treated distilled water.

Northern (RNA) analysis. Approximately 20 μg of total RNA was glyoxylated and loaded onto each lane of a 1.2% agarose gel. RNA electrophoresis and Northern transfer were carried out as described previously (33). Radioactive probes were made by using a random-priming kit (Boehringer Mannheim). Hybridization, washing, and probe removal were performed as specified by the manufacturer of the NYTRAN filter (Schleicher & Schuell, Inc., Keene, N.H.).

hGH assay. A radioimmunoassay kit for human growth hormone (hGH) was purchased from Nichols Institute. hGH secreted into culture media was assayed by the procedure suggested by Nichols Co.

CAT assay. The chloramphenicol acetyltransferase (CAT) activity was determined as described previously (6).

RESULTS

Exogenous C/EBP can repress HBV and SV40 transcription. C/EBP was shown to bind to two sites within the HBV enhancer (Fig. 1a). To see whether this C/EBP factor can activate HBV gene expression in liver cells, we cotransfected a reporter plasmid, SK, containing a tandem dimer of the wild-type HBV genome, and an effector plasmid R18, containing the cellular C/EBP gene under the transcriptional control of the RSV LTR (Fig. 1b). Almost complete inhibition of HBV-specific RNA accumulation was evident when as little as 1 μg of R18 DNA was used. Cotransfection with reporter plasmid 36, bearing a nonsense mutation in the X open reading frame of both copies of HBV, and effector plasmid R18 also resulted in significant reduction of HBV-specific RNA accumulation (Fig. 1c). These data suggest that the negative effect of C/EBP on the HBV steady-state RNA level is independent of the virus-encoded X gene product(s), which is known to be a *trans* activator (D. Pei and C. Shih, unpublished results). The C/EBP-specific transcript derived from R18 at the 2.4-kilobase position was detected when larger amounts of R18 DNA were used.

Since the C/EBP protein has been shown to recognize the enhancer domain of SV40 (Fig. 1a), we tested whether this repression phenomenon could be extended to a non-HBV system. When the reporter plasmid was substituted with an SV40 gpt vector (Fig. 1b), a similar effect of transcriptional repression was evident with the effector plasmid R18, but not with R44 (C/EBP antisense construct), which consistently demonstrated a stimulating effect on *gpt* transcription (Fig. 1d). This result suggests a more general effect of repression by C/EBP on promoter-enhancer elements containing C/EBP-binding sites.

Endogenous C/EBP can repress HBV transcription. It has been reported that in at least two different systems, overexpression of a transcriptional activator may cause inhibition of transcription (5, 37). This phenomenon, termed squelching (5), could explain the results obtained in Fig. 1c and d, considering that the RSV LTR is known to be a highly potent enhancer-promoter element.

To rule out the possibility of a squelching effect, we performed two different experiments. R44 contains the full-length C/EBP gene in an antisense orientation to the RSV LTR (Fig. 1b). No significant open reading frames on the antisense strand of the C/EBP cDNA could be identified through a computer-aided search (Pei and Shih, unpublished results).

In the first experiment, to avoid confusion by squelching, we monitored the effect of reducing the endogenous C/EBP level on HBV transcripts produced by the SK reporter. This C/EBP antisense RNA experiment was conducted with two different recipient cell lines: a human hepatoblastoma (HepG2) (1) and a rat Morris hepatoma derivative (Q7) (33; data not shown). The results obtained from both cell lines were similar. The larger the amount of R44 DNA transfected, the larger the amounts of HBV-specific transcripts that were detected (Fig. 2; see below). Hence, inhibition of endogenous C/EBP production through interference, using antisense RNA, results in increased transcription of HBV. These data strongly suggest that C/EBP functions as a negative transcriptional regulator.

The second approach to exclude squelching was to reduce the level of the endogenous C/EBP by an in vivo promoter

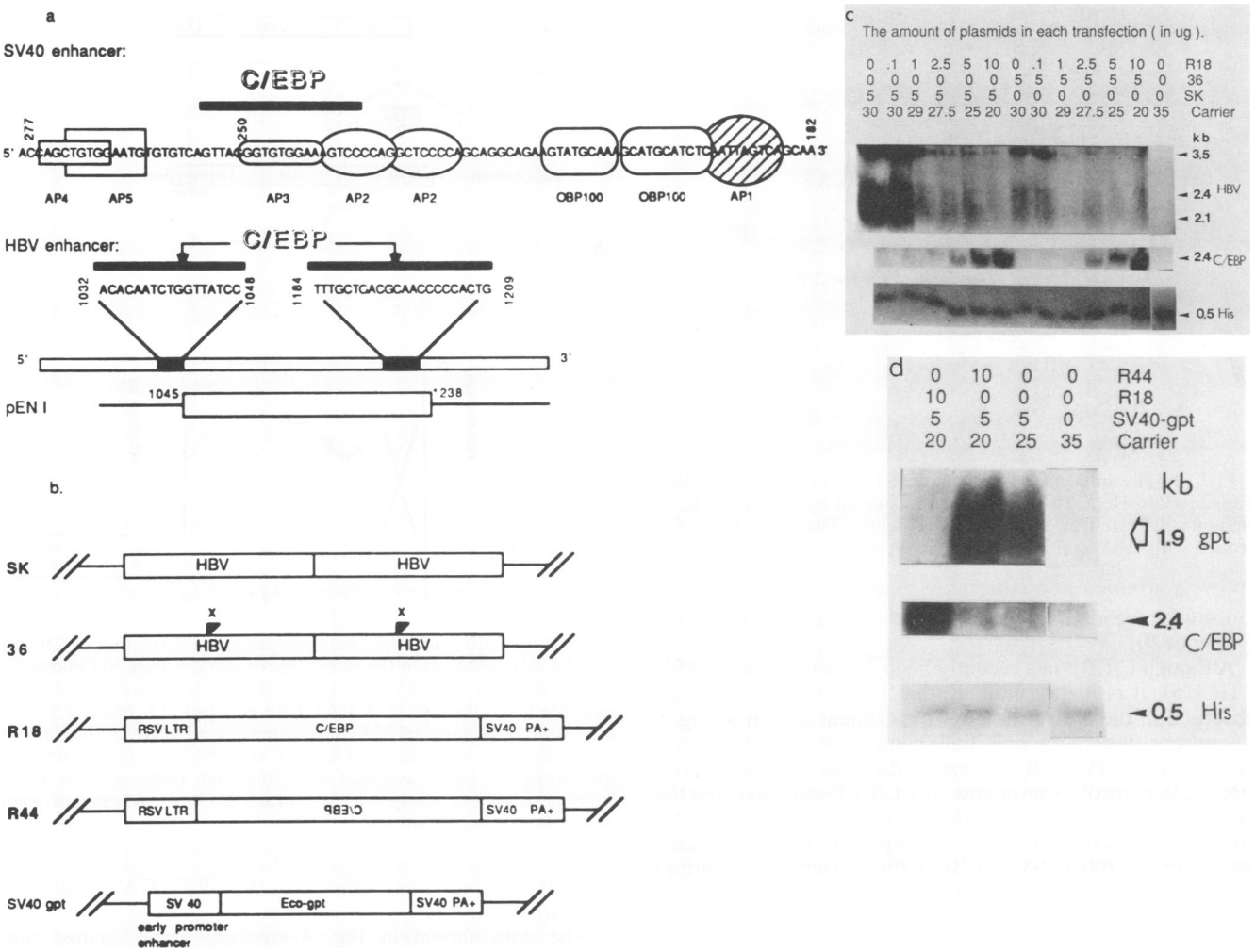

FIG. 1. C/EBP, an enhancer-promoter-binding protein of cellular origin (2, 7, 8, 12, 19, 22), behaves as a transcriptional *trans*-inactivator (or *trans*-repressor). (a) SV40 enhancer is composed of many sequence motifs to which different protein factors can bind (16); among them, C/EBP can bind to the so-called core enhancer motif (▬) (19). The HBV enhancer contains two different C/EBP sites (▬) (19). (b) Plasmids used in this work. The construction of these plasmids is described in Materials and Methods. (c) Northern analysis of HBV-specific transcripts from SK and plasmid 36 when cotransfected into HepG2 cells with increasing amounts of R18. The same filter was hybridized with three different probes: HBV, C/EBP, and His (human histone 3, a generous gift from Robert Roeder). (d) Effects of R18 and R44 on the expression of *gpt* gene under the SV40 early promoter-enhancer elements. The Northern filter was probed with vector-free, radiolabeled gpt, C/EBP, and human histone 3 DNAs.

competition experiment. We cotransfected the reporter plasmid SK with the effector plasmid pEN1 (Fig. 1a) containing the C/EBP binding site. pEN1 can stimulate HBV transcription in a dose-dependent fashion (Fig. 3). A similar effect of derepression of HBV transcription by pEN1 was observed when exogenous C/EBP was provided by cotransfection with plasmid R18.

C/EBP can activate the TK promoter. To reconcile the contradictory roles of C/EBP in transcriptional repression of SV40 and HBV enhancer-promoter demonstrated in this study with the previously proposed positive role of C/EBP on the TK promoter and liver-specific genes, we tested the hypothesis that C/EBP is able to serve as a positive or negative transcription factor depending on the individual promoter-enhancer element. We cotransfected plasmids R18 and TK-hGH (Fig. 4a). Unlike its effect on HBV and SV40, C/EBP appeared to stimulate rather than repress the TK promoter as assayed by measuring the hGH level in the medium of transfected culture (data not shown). To investigate this phenomenon further, we cotransfected an SV40 enhancer-containing plasmid, pSV232A.CAT.LRS, with plasmids R18 and TK-hGH. At various concentrations, C/EBP can function as a potent repressor for the SV40 early promoter-enhancer element as well as a powerful activator for the TK promoter. The results from the CAT assays of pSV232A.CAT.LRS are consistent with the results from RNA analysis of pSV40gpt shown in Fig. 1d.

DISCUSSION

In the experiments shown in Fig. 2, we were not able to detect any significant change of the endogenous level of the C/EBP mRNA prepared from the total population of the transfected HepG2 or Q7 cells. This is undoubtedly because only a minor fraction of the recipient cells were competent for transfection. We also were unable to detect the C/EBP antisense transcript by Northern blot analysis. We believe that this was due to either its instability in vivo or that it was

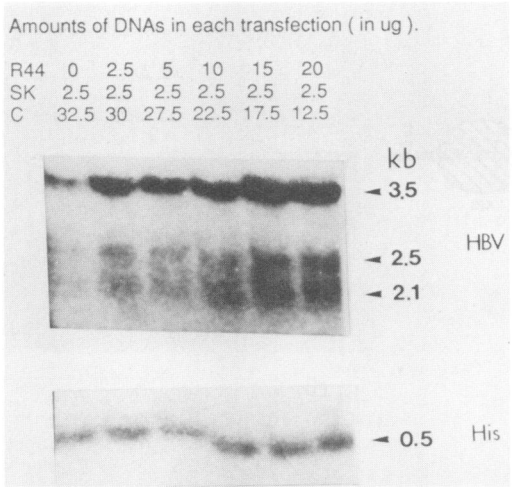

FIG. 2. The antisense construct of C/EBP, R44, can induce HBV transcription. HepG2 cells were cotransfected by R44 and SK, in amounts indicated at the top of the figure. The same filter was probed with HBV and human histone 3 (His).

too short to be identified (see the review of antisense RNA in reference 9).

Although C/EBP has recently been shown to bind to RSV LTR (28), it is highly unlikely that the derepression effect observed in the antisense RNA experiment shown in Fig. 2 is entirely due to competitive binding of C/EBP protein between the RSV LTR of plasmid R44 and the SK reporter DNAs. In control experiments, the C/EBP sequence and the pBluescript plasmid vector alone have no detectable effect on HBV transcription of the SK reporter. When the same molar dose of R44 (RSV-C/EBP in the antisense orientation) or RSV-CAT DNA was used; R44 caused derepression at

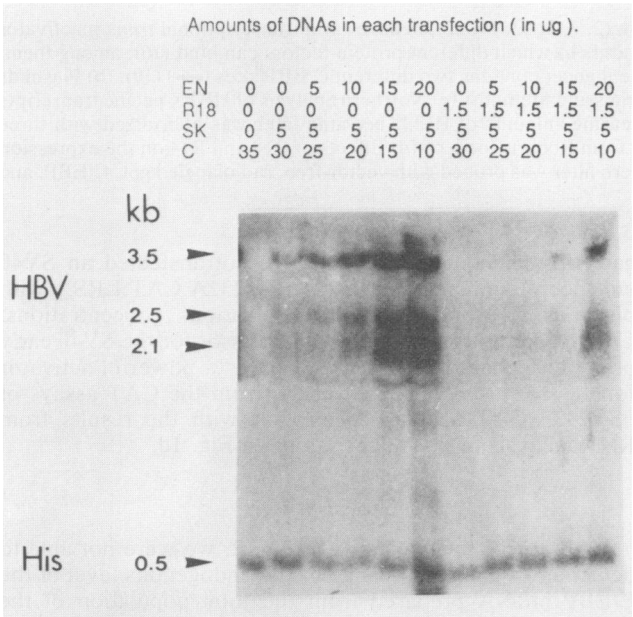

FIG. 3. The HBV enhancer can stimulate HBV transcription in *trans* and relieve the repression by C/EBP. Abbreviation: EN, pEN1. The cotransfection recipes are shown at the top of the figure. The Northern filter was probed with HBV and human histone 3 (His) as indicated.

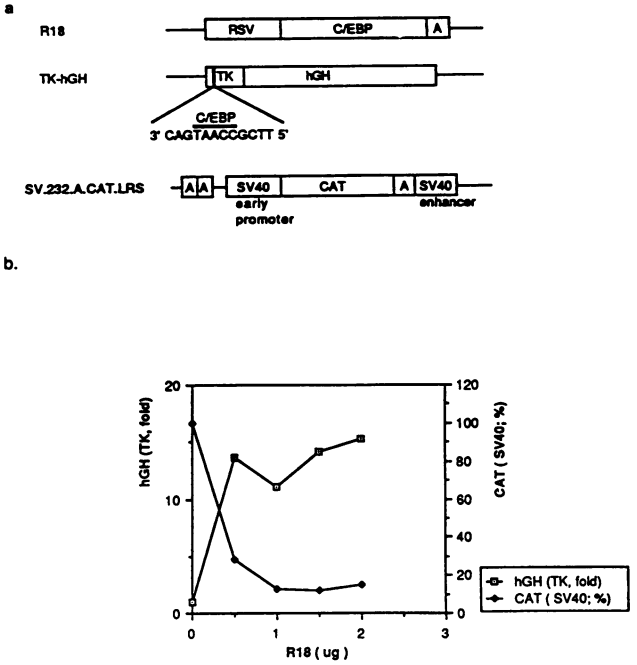

FIG. 4. Both transcriptional repression and activation of C/EBP can be demonstrated in the same experimental setting. (a) Plasmids R18, TK-hGH, and pSV232A.CAT.LRS are described in Materials and Methods. (b) pSV232A.CAT.LRS (1.5 μg), TK.hGH (0.5 μg), and various amounts of R18 were cotransfected into 5×10^5 HepG2 cells. Relative activities of TK.hGH and pSV232A.CAT.LRS are presented by being assigned as 1-fold and 100% without R18, respectively. The curves were plotted from an average of two independent experiments.

least three times more efficiently than RSV-CAT (data not shown).

The data shown in Fig. 4 strongly argue against the explanation that the repression of C/EBP is due to nonspecific cytotoxicity. Although the molecular mechanism of transcriptional activation and repression by C/EBP remains unclear, it is very likely that DNA binding of C/EBP to cognate *cis* elements is critical to transcriptional modulation. A peculiar feature of C/EBP is that it appears to bind to a wide variety of sequence motifs (Table 1). However, DNA binding per se may not be sufficient to cause activation or suppression. For example, although C/EBP has been shown to bind to the RSV LTR in vitro (28), no significant effect of C/EBP on the promoter activity of RSV LTR could be detected in vivo (Pei and Shih, unpublished results) (Table 1). The fact that HBV DNA contains two separate C/EBP-binding sites (19) will make future investigation of the repression mechanism of C/EBP on HBV more complex.

Paradoxically, although HBV is known to be a hepatotropic virus, C/EBP factor has been found to be more enriched in rat liver than in several other tissues examined (19). Perhaps another positive factor(s) (see, e.g., references 13, 32, 34, and 40) may be able to counteract the repression of C/EBP on the transcription of HBV. Different factors that can bind to the same or similar sequence motifs are not unprecedented. For example, it has been proposed that the *engrailed* repressor in *Drosophila melanogaster* can counteract the *ftz* activator by competitive interaction (14). The ultimate on-or-off decision of gene expression is most probably dependent on an averaged effect of various positive and negative interacting transcription factors existing at various

TABLE 1. Summary of the effects of C/EBP on different promoter/enhancer elements

Promoter-enhancers	C/EBP binding sequences (reference)	Effect of C/EBP
SV40	5′ TGTGGAAAT 3′ (19)	Repression
HBV	5′ TTTACACAATGTGGTTATCC 3′ 5′ AAGTGTTTGCTGACGCAACCCCCACTG 3′ (19)	Repression
TK	5′ CCAAT 3′ (8)	Activation
RSV	5′ TAGTCTTATGCAATACTCTT 3′ 5′ ACCATGTTGCAAGACTAC 3′ (28)	Neutral (data not shown)
Adenovirus E4	ND[a]	Neutral (data not shown)
mMT-1	ND	Neutral (data not shown)
Albumin, transthyretin, alpha-1-antitrypsin	5′ TCNTACTC 3′ (2) (consensus)	ND

[a] ND, Not determined.

concentrations. Another possibility is that the heterogeneity of hepatocytes in the liver (10) is correlated with a different content of C/EBP and thus confers a different degree of susceptibility to HBV infection. Finally, whether the liver tissue in humans is also the most abundant tissue containing C/EBP protein, as it appears to be in rats, remains to be investigated. Although tempting, it appears too speculative at this point to propose that chronicity and latency of viral infection and repressor activity of C/EBP are related.

The capability of C/EBP to function as both a positive and a negative factor and the intriguing property of its low degree of binding specificity in vitro suggest that it regulates a large number of cellular genes during development and growth control, similar to the glucocorticoid receptor in mammalian systems (26). Interestingly, our recent results have indicated that the activation or repression effect of C/EBP can be extended to nonviral genes in a nonhepatocyte system (Pei and Shih, unpublished results).

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