MOLECULAR AND CELLULAR BIOLOGY, Feb. 1986, p. 710–715
0270-7306/86/020710-06$02.00/0

Vol. 6, No. 2

The Human Hepatitis B Virus Enhancer Requires *trans*-Acting Cellular Factor(s) for Activity

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Received 25 July 1985/Accepted 1 November 1985

The activity of the hepatitis B viral enhancer element was studied in various cell lines. This enhancer shows strict host and tissue specificity in that it is functional only in liver cells of human origin. Further, it requires *trans*-acting factor(s) present in liver cells for activity, and this activity is independent of hepatitis B virus gene products in the cell lines tested.

The human hepatitis B virus (HBV) elicits acute and chronic liver disease in humans and has been linked to hepatocellular carcinoma (19, 26). Its narrow host range and inability to grow in culture may be attributed to specific liver tropism. One class of DNA regulatory elements, the enhancers, that exhibit tissue/host-specific action have been reported in a number of viral genomes and cellular genes (1–3, 6, 9, 13). Enhancers are able to activate the transcription of native as well as heterologous genes relatively independently of distance and orientation with respect to the coding region (2, 5, 16). HBV enhancer sequences were recently reported in the region of nucleotides 1000 to 1250 on the HBV map, about 400 base pairs upstream of the core promoter, between the coding sequences of the surface antigen (HBsAg) gene and the "X" open reading frame (22). The contrast between the weak core promoter activity in nonprimate or nonhepatic cell types and the production of large amounts of core antigen (HBcAg) during HBV infection in humans prompted us to investigate the influence of HBV enhancer sequences on this promoter. To quantitatively study the enhancer-mediated activity of the core promoter, we used the assayable chloramphenicol acetyltransferase (CAT) system (7). Such a system has been extensively used to study expression under the control of heterologous promoters, enhancers, or both (6, 7).

Figure 1 outlines the scheme of construction of the CAT recombinant plasmids. Plasmid pCPCAT contains HBV sequences between the *Acc*I (nucleotide 1076) and *Bgl*II (nucleotide 1991) sites linked to the CAT gene. The HBV fragment present in pCPCAT contains an uninterrupted sequence that includes the HBcAg promoter (17) as well as the enhancer (22). Plasmid pCPCAT-ΔE contains the HBcAg promoter, but the enhancer sequences are deleted. These plasmids were transfected and transiently expressed in HepG2 human hepatoma cells (HBV negative) (10). The result (Fig. 2A) shows a marked reduction in CAT activity by the core promoter when the 5′ enhancer sequences were removed. This suggests that the HBcAg promoter requires upstream sequences for efficient expression. Similar results were obtained in Hep3B human hepatoma cells (HBV positive) (10) (data not shown).

Enhancer sequences usually exhibit maximal activity in their natural host cells and show tissue specificity (11). Similarly, the HBV enhancer was previously shown to be functionally active in a human hepatoma cell line (PLC/PRF/5) and not in CV-1 cells. However, since the PLC cells contain at least seven integrated copies of HBV DNA and constitutively secrete HBsAg (15, 23), the nature of the interaction(s) of HBV enhancer sequences in liver cells remains to be understood. To study the host and tissue specificity of this enhancer, we compared the expression of plasmid pCPCAT in various cell lines: rat fibroblasts (APB), rat hepatoma cells (FaO, 4C2), mouse hepatoma cells (HEPA-1), monkey kidney fibroblasts (CV-1), human foreskin fibroblasts (FS24), HeLa cells, and two human hepatoma cell lines, HepG2 and Hep3B. The human hepatoma cell lines used in this study are physiologically identical for at least a dozen markers, except that Hep3B contains integrated HBV sequences and constitutively secretes HBsAg, whereas HepG2 is devoid of HBV sequences and therefore produces no viral markers (10). Different cell lines were transfected with 20 μg of pCPCAT DNA using the calcium phosphate coprecipitation method (7, 8), and cell lysates were assayed for CAT activity (7) 48 h posttransfection. Results of the CAT expression in various cell lines are summarized in Table 1 and shown in Fig. 2B. It is clear that the HBV enhancer-mediated expression is markedly higher in human cells of hepatic origin. All cell lines were independently transfected with plasmid pSV2CAT (contains the simian virus 40 [SV40] enhancer) as a control, which showed maximal activity in CV-1 cells (Table 1). The HBV enhancer exhibits preferential expression in human liver cells but not in nonhuman liver cells (FaO, 4C2, and HEPA-1 cells; Fig. 2B and Table 1) nor in human cells of nonhepatic origin (FS24 and HeLa cells; Fig. 2B and Table 1).

The presence and expression of HBV sequences in the hepatoma cell line and their role in *trans*-activation of the HBV enhancer appear to be neutral since no significant difference in pCPCAT expression was found between HepG2 and Hep3B human hepatomas (Fig. 2B). To confirm this result using an independent approach, we cotransfected HepG2 cells with pCPCAT and with plasmid vectors capable of expressing HBV gene products. Plasmids pNEP and pNET contain the genome-length HBV (*adw2*) DNA cleaved at *Apa*I (nucleotide 2412) and *Taq*I (nucleotide 2020) sites, respectively, inserted in pML-*neo* (pML-*neo* was constructed by inserting the neomycin gene in plasmid pML [14]) and express HBsAg (A. Siddiqui, S. Jameel, and J. Mapoles, Proc. Natl. Acad. Sci. USA, in press). Plasmid pNEC contains a 1.85-kilobase *Bam*HI fragment of HBV (nucleotides 1407 to 34), also in pML-*neo*, and expresses

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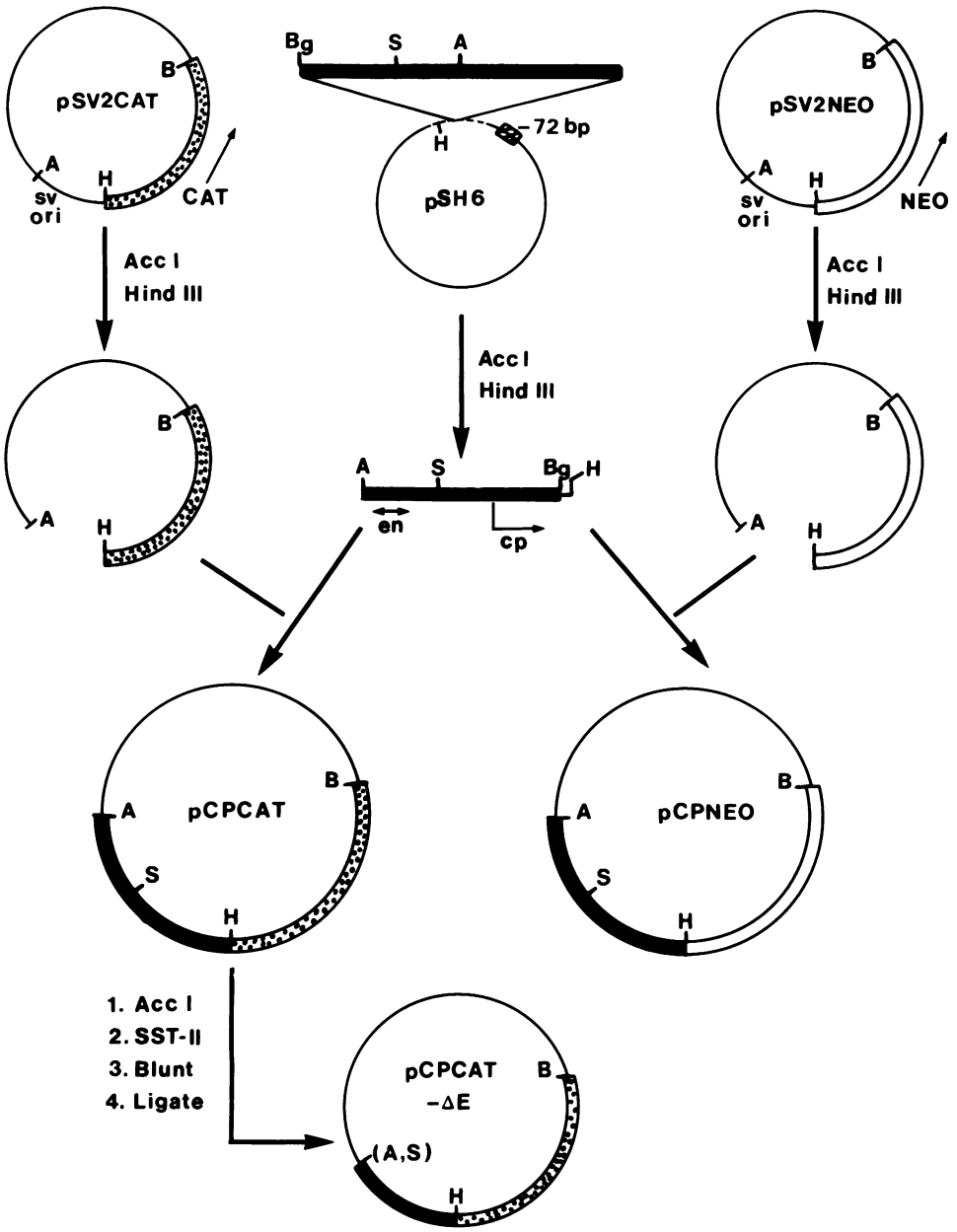

FIG. 1. Construction of recombinant plasmids. Plasmid pSH6 (24), which contains the 2.8-kilobase *Bgl*II fragment of HBV in pSV010 (12), was cleaved with *Acc*I and *Hin*dIII to isolate a 0.93-kilobase HBV fragment (map position, nucleotides 1076 to 1991). This fragment, containing the HBV enhancer (en) and the HBcAg promoter (cp), was inserted upstream of either the CAT gene or the *neo* gene to yield pCPCAT or pCPNEO, respectively. The CAT and *neo* genes were part of plasmids pSV2CAT and pSV2NEO, respectively, and contain the SV40 t splice and polyadenylation sequences 3′ to the coding region (7, 25). Both pSV2CAT and pSV2NEO were cleaved with *Acc*I and *Hin*dIII to remove the SV40 *ori* sequences before the HBV 0.93-kilobase fragment was inserted. Plasmid pCPCAT-ΔE was constructed by digesting pCPCAT with *Acc*I and *Sst*II (HBV map position, nucleotides 1076 to 1458), which deletes the enhancer sequences, leaving the HBcAg promoter 5′ to the CAT gene. A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; S, *Sst*II.

HBc/eAg (M. Roossinck, S. Jameel, S. H. Loukin, and A. Siddiqui, submitted for publication).

HepG2 cells were contransfected with pCPCAT and an excess of the HBV plasmid vectors (a 3.6-fold molar excess of pNEP or pNET; a 4.3-fold molar excess of pNEC). No further stimulation of CAT expression was seen as a result of this cotransfection (Fig. 2C). When tested (18), the extracts showed the presence of neomycin phosphotransferase activity, suggesting that cells had received the pML-*neo*-based HBV plasmid vectors (data not shown). The culture supernatants were also subjected to commercial radioimmunoassays (RIAs; Abbott Laboratories) which detect HBsAg or HBc/eAg (Fig. 2C). Taken together, these results show that neither the presence nor the expression of HBV sequences mediates activation of the HBV enhancer element. We further confirmed this result by transient expression of plasmid pCPCAT in stably transformed rat cells continually expressing either HBsAg and X or HBcAg (data not shown).

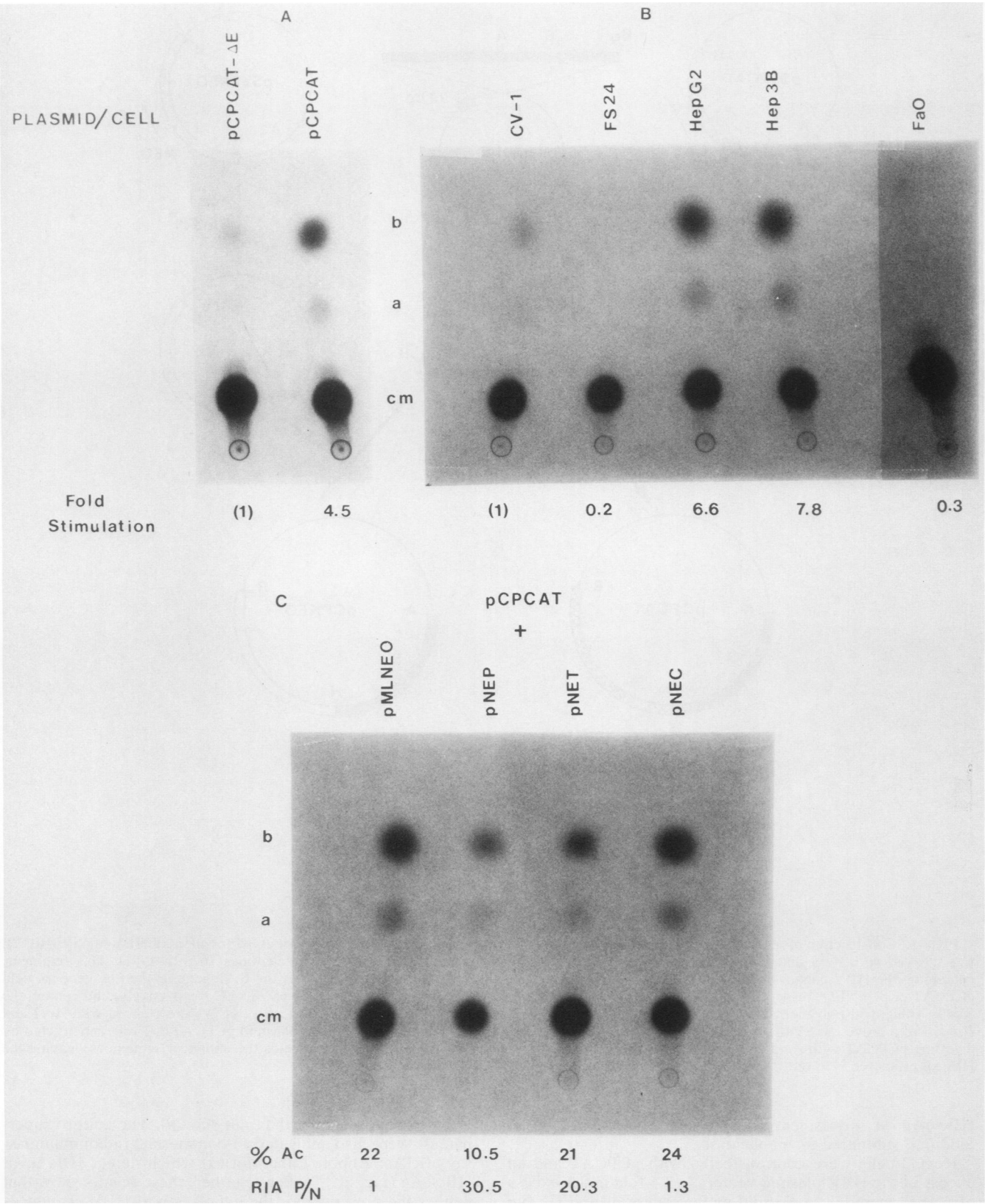

FIG. 2. (A) CAT activity of HepG2 cells transfected with either pCPCAT-ΔE or pCPCAT DNA. Fold stimulation was normalized with respect to pCPCAT-ΔE expression. (B) CAT activity of CV-1, FS24, HepG2, Hep3B, and FaO cells transfected with pCPCAT DNA. Fold stimulation was normalized with respect to pCPCAT expression in CV-1 cells. (C) CAT activity in HepG2 cells cotransfected with 3.5 μg of pCPCAT DNA and 21.5 μg of either pML-*neo*, pNEP, pNET, or pNEC DNA. The RIA positive/negative (P/N) ratios are for 48 h

The CAT expression by pCPCAT in these cell lines was similar to that seen in untransformed rat cells.

For some enhancers, the cell type specificity has been attributed to a specific interaction(s) between the enhancer and one or more factors present in the specific cells (20, 21). To determine whether such cell-specific molecules or factors exist in liver cells and interact with the HBV enhancer, we carried out an in vivo titration study (21). The minimum amount of pCPCAT DNA required for optimal expression in HepG2 cells was found to be 1 pmol (3.5 μg) (Fig. 3A). Plasmid pCPCAT showed low expression in the presence of 0.56 pmol (2.0 μg), followed by increased expression with increasing amounts of specific DNA used; the CAT signal leveled off beyond about 2 pmol (7.0 μg) of DNA, suggesting a saturation phenomenon. Although this is not the only explanation, this saturation could be caused by limiting amounts of cellular factor(s) required for enhancer activity. If this is true, it should be possible to titrate out such a cellular factor(s) by using a competitor plasmid that contains the same transcriptional control signals (enhancer-promoter unit) as pCPCAT but encodes a different activity. For this we used the plasmid pCPNEO (Fig. 1). One picomole (3.5 μg) of pCPCAT DNA was cotransfected with increasing amounts of pCPNEO DNA into HepG2 cells. The total amount of DNA used per transfection was kept constant with pBR322 DNA. Figure 3B shows that increasing amounts of pCPNEO decreased the CAT signal obtained from a constant amount of pCPCAT. The possibility that decreased CAT expression was due to decreased DNA uptake was eliminated by assaying for neomycin phosphotransferase activity in cell extracts. Plasmid pCPNEO showed a dose response similar to that of pCPCAT in HepG2 cells. To ascertain whether the factor present in HepG2 cells is specific for the HBV enhancer or for a variety of other enhancer sequences as well, we carried out the competition with heterologous enhancers. When plasmid pSV2NEO, which contains the SV40 enhancer, was used as the competitor DNA, a reduction in the CAT was seen, although not as much as with pCPNEO (Fig. 3B). A similar reduction in the CAT signal was also seen with plasmid pRSVNEO, which contains the Rous sarcoma virus long terminal repeat (data not shown). These results, taken together with the saturation effect seen with increasing amounts of pCPCAT DNA, point towards an interaction between the HBV enhancer and a factor(s) present in human liver cells. Although the nature of this interaction is not limited to the HBV enhancer, there appears to be a higher degree of specificity for these sequences as compared to heterologous enhancers, i.e., SV40 and Rous sarcoma virus. Whether the factor(s) responsible belongs to a class of transcription factors that interact specifically with enhancer sequences remains to be determined.

The present study clearly demonstrates the interaction of a *trans*-acting cell-specific factor(s) with the HBV enhancer element. This enhancer is required for efficient transcription from the HBcAg promoter, in agreement with earlier observations (22). Other work from our laboratory involving the expression of the HBcAg gene under its natural transcriptional controls also supports this observation (Roossinck et al., submitted). When plasmids pNEC and pCEN (pCEN contains a *Hpa*I-to-*Eco*RI fragment of HBV that includes 5′ flanking enhancer sequences, whereas pNEC lacks the enhancer) were transiently expressed in HepG2 cells, the latter showed at least 10- to 15-fold higher expression of HBc/eAg compared to the former. Thus, the reporter CAT gene used in our experiments supports the interpretation regarding natural transcriptional controls of the HBcAg gene. Furthermore, the enhancer element may regulate the synthesis of longer than genome-length pregenomic RNA species which have been found to be transcribed from the HBcAg promoter in infected livers (4). The presence of a cellular factor(s) in liver cells with affinity for and the capability to *trans*-activate the HBV enhancer may explain the hepatotropism of this virus. This may also provide a model for the role of HBV in establishing primary hepatocellular carcinoma in humans. Although infection by HBV has been suggested as an important event in the development of this disease, a definite mechanism by which HBV mediates oncogenesis remains to be established (27). None of the viral genes has been shown to be linked with a transformation function. It has been proposed that the transforming potential of certain leukemia viruses that lack oncogenes is due to enhancer-mediated activation of cellular genes (13). A recent report has corre-

TABLE 1. CAT activity in cells after transfection with pCPCAT, pCPCAT-ΔE, and pSV2CAT DNA

Cell line	Origin	CAT activity[a]		
		pCPCAT	pCPCAT-ΔE	pSV2CAT
CV-1	Monkey kidney (epithelial)	(+)	ND	++
FS24	Human foreskin fibroblasts	−	ND	ND
HeLa	Human cervix carcinoma	−	−	+
HepG2	Human hepatoma, HBV negative	+++	+	+
Hep3B	Human hepatoma, HBV positive	+++	−	+
PLC/PRF/5	Human hepatoma, HBV positive	++	ND	+
APB	Rat fibroblasts	+	NA	+
Fa0	Rat hepatoma	−	NA	+
4C2	Rat hepatoma	+	ND	+
HEPA-1	Mouse hepatoma	+	−	+

[a] The percentage of acetylated chloramphenicol in CV-1 cells transfected with pCPCAT DNA was taken as the denominator, and enhancement factors were calculated accordingly. +, No enhancement; ++, two to five-fold enhancement; +++, greater than fivefold enhancement; −, less than denominator; NA, no detectable activity; ND, not done.

posttransfection culture supernatants. Each assay contained 0.5 mg of protein (BioRad assay) and was carried out for 2 h at 37°C. After separation of the reaction products by ascending thin-layer chromatography (0.2-mm silica gel) and visualization by autoradiography (Kodak XAR-5 film), the assay was quantitated by counting regions of the gel containing unacetylated [^{14}C]chloramphenicol (cm) and its 1-acetate (a) and 3-acetate (b) forms. The RIAs were carried out with an Ausria II RIA kit for HBsAg and a similar kit for HBeAg (Abbott Laboratories). Before the RIA, culture supernatants were clarified by centrifugation at 12,000 × *g* for 30 min. For HBsAg RIA, the particles were pelleted in a Beckman SW 55 rotor at 45,00 rpm for 4 h and the pellets were resuspended in 350 μl of phosphate-buffered saline (per 100-mm plate), of which 200 μl was used for the RIA. For HBc/eAg RIA, the clarified supernatants were concentrated 35-fold with an Amicon YM-2 filter and 200 μl was used for the RIA. The controls included HBsAg-positive/negative (P/N = 30.9) and HBc/eAg-positive/negative (P/N = 4.9) human plasma samples.

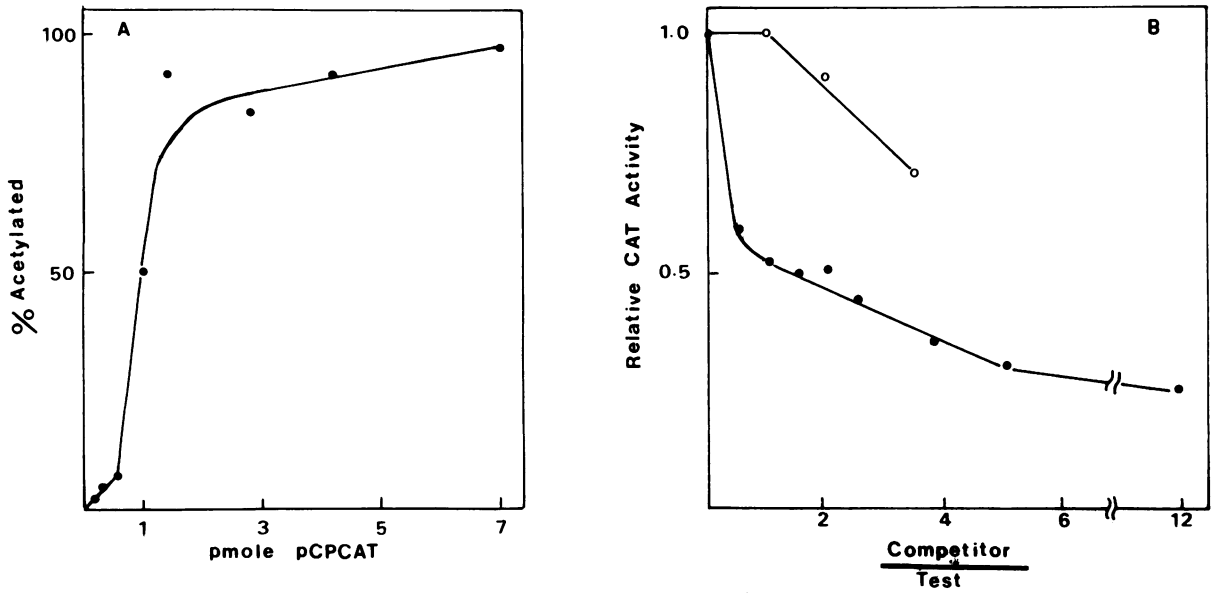

FIG. 3. Competition assay for enhancer activity. (A) CAT activity of HepG2 cells transfected with increasing amounts of pCPCAT DNA. (B) CAT activity of HepG2 cells cotransfected with 1 pmol (3.5 μg) of pCPCAT DNA (Test DNA) and increasing amounts of competitor DNA: pCPNEO (●) or pSV2NEO (○). The titrations were carried out as described by Scholer and Gruss (21), except that all transfections were carried out with 25 μg of total DNA made up with pBR322. The relative CAT activities were normalized to a transfection containing 1 pmol (3.5 μg) of pCPCAT DNA and 21.5 μg of pBR322 DNA (acetylated [^{14}C]chloramphenicol, 56.1%). In one case when 11.6 pmol of pCPNEO was used, the total amount of DNA in the transfection was 50 μg. The CAT activity for this was normalized with respect to a transfection containing 1 pmol (3.5 μg) of pCPCAT DNA and 46.5 μg of pBR322 DNA (acetylated [^{14}C]chloramphenicol, 45%).

lated the enhancer activity with the oncogenic potential of certain avian retroviruses (28). Although it is probable, it remains to be determined whether the HBV enhancer can mediate hepatocarcinogenesis by this mechanism.

We thank B. Knowles for the Hep G2 and Hep 3B cell lines, B. H. Howard for plasmid pSV2CAT, J. Mapoles for comments, and S. Loukin and S. K. Siddiqui for technical assistance.

This work was supported by Public Health Service grant CA-33135 from the National Institutes of Health to A.S.

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