JOURNAL OF VIROLOGY, Aug. 1990, p. 3977–3981
0022-538X/90/083977-05$02.00/0

Vol. 64, No. 8

# A New Enhancer Element, ENII, Identified in the X Gene of Hepatitis B Virus

YUAN WANG,* PING CHEN, XUE WU, AI-LONG SUN, HENG WANG, YU-AO ZHU, AND ZAI-PING LI

*Shanghai Institute of Biochemistry, Academia Sinica, Shanghai 200031, People's Republic of China*

Received 4 January 1990/Accepted 2 May 1990

**A new enhancer element of hepatitis B virus HBV, ENII, located in the X gene coding region and upstream of the C promoter, has been identified. As determined by deletion analysis, the sequence around nucleotides 1627 to 1732 was suggested to be essential for ENII activity. ENII was cell type specific. It showed high activity in HepG2 cells but no detectable activity in CV-1 cells. A protein-binding site was identified by footprinting in nucleotides 1648 to 1671. The minimum sequence and function of ENII are under investigation.**

Hepatitis B is a serious worldwide viral disease caused by hepatitis B virus (HBV). Chronic infection by HBV is closely related to the development of hepatocellular carcinoma. Although HBV vaccine has been proven to be useful in protecting from HBV infection, there are no efficient therapeutic measures for the control of HBV. Study of the expression and regulation of HBV genes is one of the most important approaches in looking for new breakthroughs.

HBV has a double-stranded DNA genome with a partially single stranded region that codes for surface antigen (S gene), core antigen (C gene), X protein (X gene), and viral polymerase (P gene) (10). Four promoters have been identified in HBV: promoters of the S gene, SPI and SPII (7), C promoter (4, 5), and X promoter (12). An enhancer has also been identified in HBV (6, 11), which is capable of stimulating the transcriptional function of HBV promoters. Another enhancer element associated with the glucocorticoid receptor-binding site was shown to be present in the S gene coding region (13). The X gene product also positively regulates various promoters, but in a *trans*-acting manner (8, 17). Investigation of the regulatory elements and their biological function is crucial to an understanding of viral gene expression.

We report here our results on the identification of a new enhancer element, ENII, which is located in the X gene coding region and upstream of the C promoter.

Plasmid pADR-1 contains a complete genome of HBV subtype adr (15), which has a unique *Bam*HI site downstream of the S gene. The HBV genome cloned in pADR-1 is 3,215 base pairs (bp) in size and has been completely sequenced (1). By cleavage at the *Bst*EII site upstream of the pre-S1 region of the S gene followed by insertion of a *Bam*HI linker, a new *Bam*HI site was introduced in place of the *Bst*EII site. The resulting plasmid, pADR-2B, has two *Bam*HI sites. HBV DNA of pADR-2B was divided into two parts by *Bam*HI digestion. One fragment of 1.8 kilobase pairs (kb) contains the pre-S1–pre-S2–S coding sequence, the SPII promoter, the glucocorticoid receptor-binding element site, and the previously mapped enhancer element; the second fragment of 1.4 kb contains the X gene, the C gene, and the upstream sequence of pre-S. These two fragments were inserted into plasmid pA10CAT (Fig. 1), which contains a simian virus 40 (SV40) early promoter in front of the chloramphenicol acetyltransferase (CAT) gene. There is no 72-bp enhancer sequence in pA10CAT, which is therefore incapable of any significant CAT gene expression. The derived plasmids pA10CATB1.4 and pA10CATB1.8 were used to transfect HepG2 cells, a highly differentiated hepatocellular carcinoma cell line. Interestingly, when these two HBV DNA fragments were placed downstream of the CAT gene, marked elevation of CAT activity was observed (Fig. 2). pSV2CAT, in which efficient expression of the CAT gene is directed by both the early promoter and enhancer of SV40 in front of the CAT gene, was used as the positive control. Enhancement of CAT activity in pA10CATB1.8 was as expected, since within the 1.8-kb fragment there is an enhancer element of HBV previously mapped downstream of the S gene. The distinct CAT activity of pA10CATB1.4, on the other hand, suggested the presence of a novel enhancer element within the 1.4-kb HBV fragment.

The 1.4-kb HBV fragment was further digested with *Bgl*II into three fragments. The *Bam*HI-*Bgl*II fragment (1403 to 1987) contains the X protein-coding region, the core promoter, pre-core region, and the N-terminal of the core protein. The *Bgl*II-*Bgl*II fragment (1988 to 2426) contains the middle portion of the core antigen-coding sequence. The *Bgl*II-*Bam*HI (*Bst*EII) fragment (2426 to 2818) contains the C terminus of the core antigen and the promoter SPI sequence of the pre-S1 gene (Fig. 3). These three fragments were inserted into the *Bam*HI site of pA10CAT, producing pA10CATBx1, pA10CATBc, and pA10CATBr, respectively. Upon transfection of HepG2 cells, only pA10CATBx1, which contains the 584-bp *Bam*HI-*Bgl*II fragment, could stimulate SV40 promoter and enhanced CAT activity (Fig. 4). Furthermore, the stimulatory activity was similar when this fragment was inserted into the *Bgl*II site of pA10CAT upstream of the SV40 early promoter in different orientations, as shown in Fig. 4 for pA10CATBx2 and pA10CATBx3. As mentioned, the *Bam*HI-*Bgl*II (1403 to 1987) fragment includes most of the X protein region. However, involvement of the X protein residues as *trans*-acting factors in enhancing CAT activity was unlikely since the constructs lacked the X promoter and the first ATG of the X open reading frame. Furthermore, the *Bam*HI-*Bgl*II (1403 to 1987) fragment was active in a position- and direction-independent manner. It is one of the characteristic features of enhancer elements. Our results demonstrate the presence of a new enhancer element, ENII, within the *Bam*HI-*Bgl*II site (1403 to 1987) of the HBV genome.

The HBV enhancer defined by Shaul et al. (6) showed high tissue specificity. It functioned much better in the highly differentiated hepatoma cells but not in nonliver cells or

* Corresponding author.

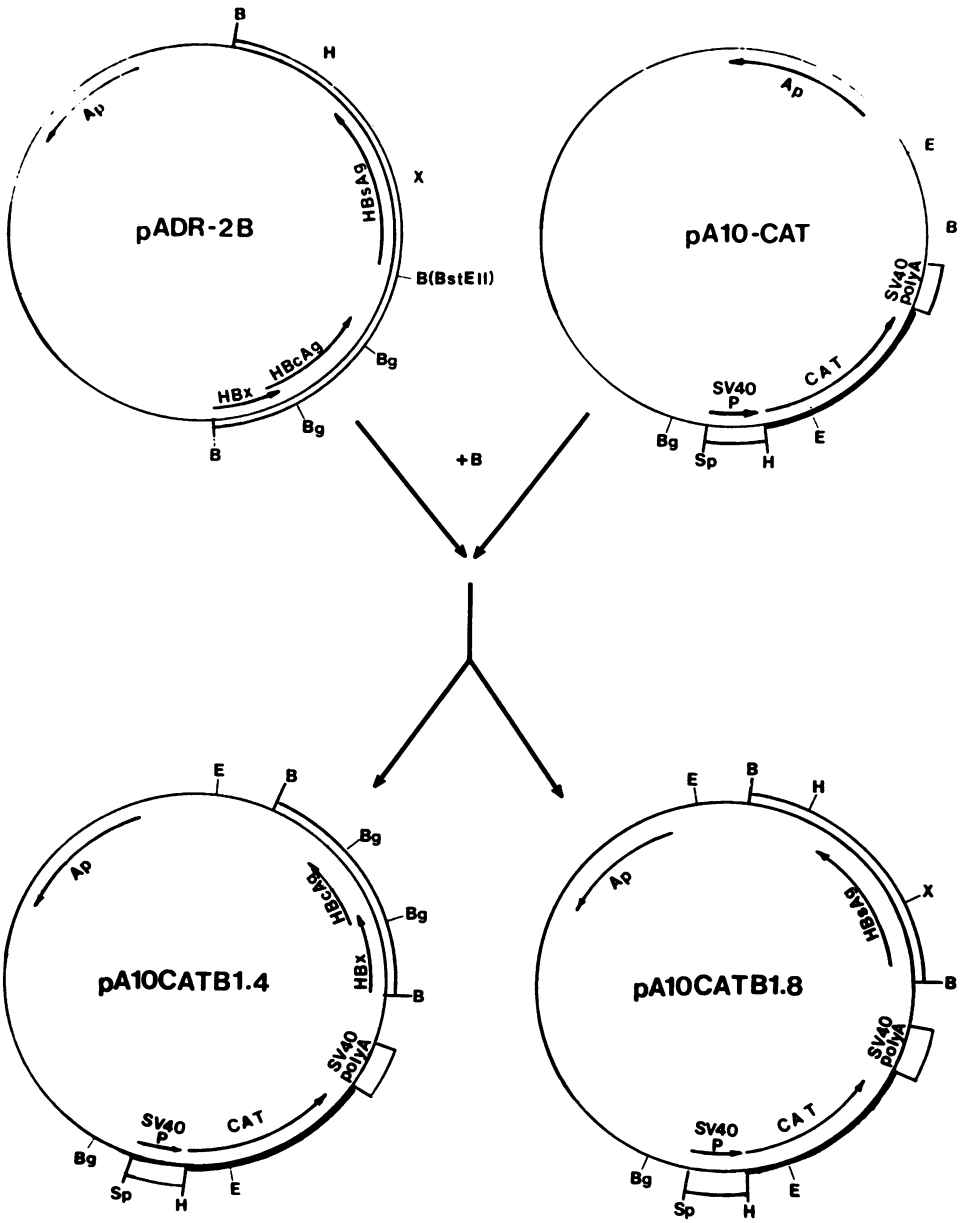
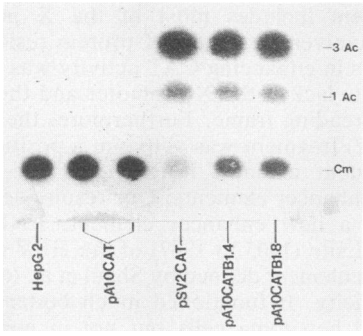



FIG. 1. Construction of CAT plasmids pA10CATB1.4 and pA10CATB1.8. In pADR-2B, a *Bam*HI site was introduced in place of the *Bst*EII site upstream of the HBV surface antigen (HBsAg) gene. The 1.4- and 1.8-kb HBV fragments were inserted downstream of the CAT gene in pA10CAT. HBcAg, HBV core antigen gene.




undifferentiated hepatoma cells (4–6). On the other hand, there are experimental results showing this HBV enhancer-activated transcription to be relatively cell type independent (14). To examine whether this new enhancer also works cell type specifically, plasmids pA10CATBx1, pA10CATBx2, and pA10CATBx3 were transfected into HepG2 and CV-1

FIG. 2. CAT assay for pA10CATB1.4 and pA10CATB1.8 in HepG2 cells. A 10-μg amount of each plasmid was introduced into cells by the method of calcium phosphate-mediated coprecipitation. CAT assays were performed 48 h after transfection as described by Gorman et al. (2). pSV2CAT and pA10CAT were used as the positive and negative controls, respectively.

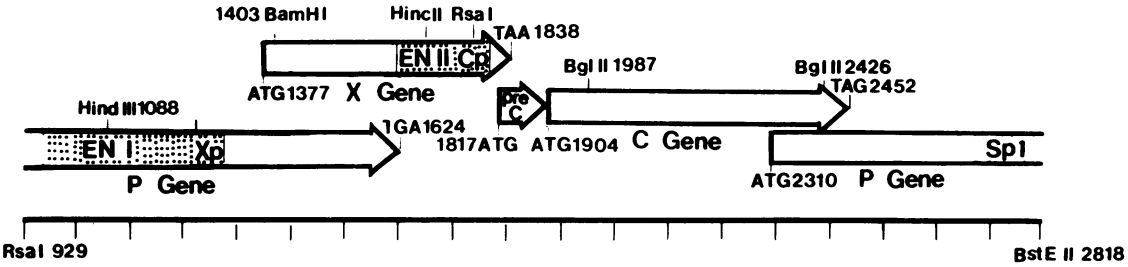



FIG. 3. HBV genome structure between *Rsa*I and *Bst*EII (929 to 2818). Open boxes represent the relative genes. Promoters and initiation and termination codons of the X, C, and P genes are indicated, as are the important restriction sites. The *Bst*II site has been changed to *Bam*HI as described in the legend to Fig. 1. All of the nucleotide numbers given in this report were assigned by taking the first nucleotide of the mutated recognition sequence of *Eco*RI as nt 1, since there is no *Eco*RI site within HBV DNA of pADR-1 (1, 14).

cells simultaneously. The new enhancer element, ENII, in these three plasmids enhanced CAT gene expression in HepG2 cells (Fig. 4) but not in CV-1 cells (Fig. 5), thus demonstrating the cell type specificity of this new enhancer element. The function of ENII in HeLa cells, (nonliver human cells) was also examined; no detectable CAT activity was found (data not shown). The proximity of ENII to the C promoter suggests an essential role for the highly cell type dependent transcriptional activity of the C promoter (9).

To pinpoint the location of this new enhancer element, deletion analysis was done by BAL 31 digestion of plasmid pA10CATBx1 from the *Bam*HI site (Fig. 6). It was found that deletion of up to 224 bp of *Bam*HI-*Bgl*II fragment from the *Bam*HI site, i.e., deletion to nucleotide (nt) 1627, did not influence its function in enhancing CAT activity. However, further deletion to position nt 1732 greatly abolished the enhancer activity. These data suggested that the sequence between nt 1627 and 1732 is essential for ENII enhancer activity. A 147-bp fragment (1627 to 1774) was inserted upstream of the SV40 promoter, and it functioned as well (Table 1).

To rule out the possibility that this region acts in *trans* via

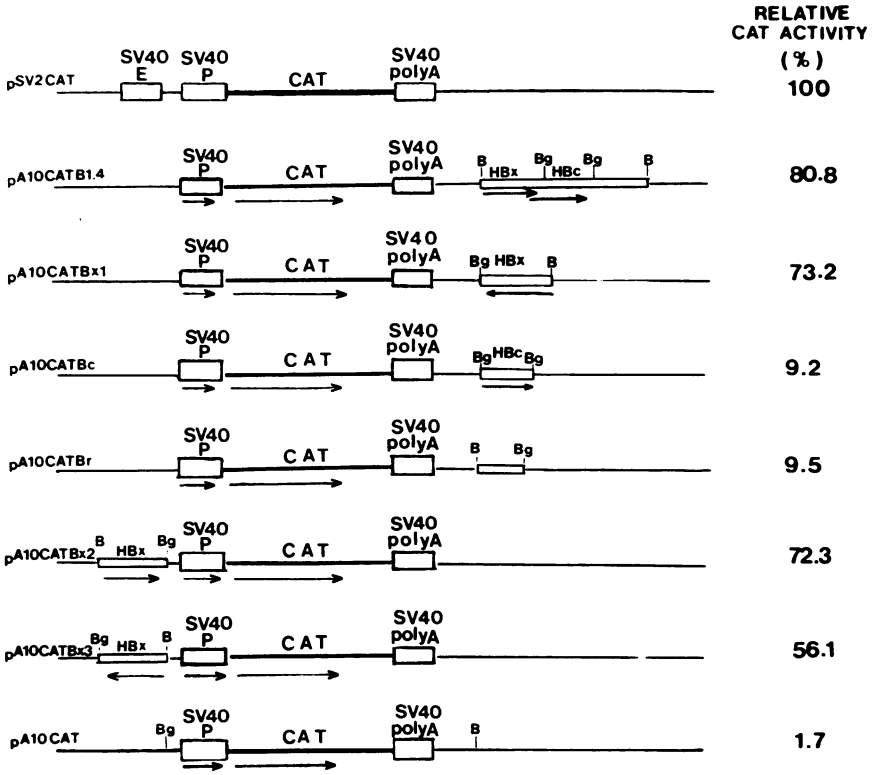



FIG. 4. Structures and relative CAT activities of plasmid constructs. HepG2 cells were transfected as described in the legend to Fig. 1. HBV DNA fragments harboring the ENII enhancer element were inserted into the downstream *Bam*HI site or the upstream *Bgl*II site of the CAT expression unit of pA10CAT. The *Bam*HI-*Bam*HI (*Bst*EII) 1.4-kb fragment in pA10CATB1.4 was the HBV DNA from nt 1403 to 2818. The *Bam*HI-*Bgl*II HBx fragment in pA10CATBx1, pA10CATBx2, and pA10CATBx3 was the HBV DNA from nt 1403 to 1987. The *Bgl*II-*Bgl*II HBc fragment in pA10CATBc was the HBV DNA from nt 1988 to 2426. The *Bgl*II-*Bam*HI (*Bst*EII) fragment in pA10CATBr was the HBV DNA from nt 2427 to 2818. CAT activity was determined by densitometrically scanning of autoradiographs and expressed relative to the activity of pSV2CAT, taken as 100. Values are means of three independent experiments.

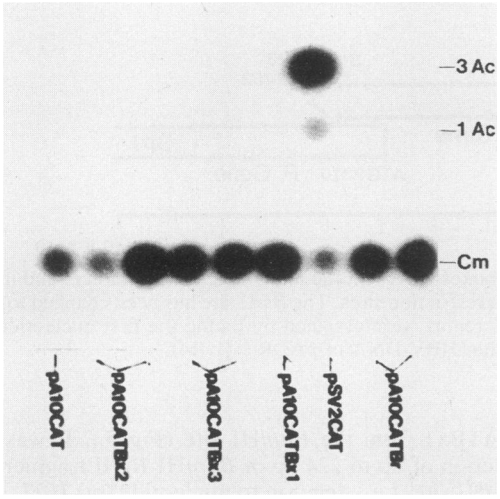



FIG. 5. Expression of CAT activity in CV-1 cells. CV-1 cells were transfected with the indicated plasmids. CAT assays were done as described in the legend to Fig. 1.

the synthesis of a truncated X gene product, a cotransfection experiment was performed. The *Bam*HI-*Stu*I (1403 to 2024) fragment and a shorter fragment (1627 to 1879) were cloned into vector Bluescribe (Genefit product), and the resulting plasmids BlueBx and BlueBx23A were cotransfected with pA10CAT. No significant CAT activity was observed (Table 1).

The *trans*-regulating factors that may be required for the function of the new enhancer were also studied. Nuclear extracts from HepG2 cells were prepared. Band shift and competition assay were performed, using the *Bam*HI-*Bgl*II fragment (1403 to 1987) and a shorter fragment *Bam*HI-

TABLE 1. Relative CAT activity via cotransfection

| Construct[a] | Relative CAT activity[b] |
|---|---|
| pA10CAT | 1.0 |
| pA10CATBx1 | 21.6 |
| pA10CATBx23R | 57.5 |
| pA10CAT + BlueBx | 0.47 |
| pA10CAT + BlueBx23A | 3.7 |

[a] pA10CATBx1 contains the *Bam*HI-*Bgl*II (1403 to 1987) fragment of HBV downstream of the CAT gene, pA10CATBx23R contains a shorter fragment (1627 to 1774) of HBV upstream of the SV40 promoter, BlueBx has the *Bam*HI-*Stu*I (1403 to 2024) fragment of HBV inserted into the vector Bluescribe (Genefit product), and BlueBx23A has the fragment of HBV from nt 1627 to 1879 inserted into Bluescribe. BlueBx and BlueBx23A were cotransfected with pA10CAT as described in the text.

[b] Expressed as fold enhancement over pA10CAT activity. Values are means of three independent experiments.

*Hin*cII (1403 to 1685). The results revealed the presence of a sequence-specific binding protein in the nuclear extracts that bound to the sequence where the new enhancer element is located. DNase I footprinting analysis showed the binding site of this protein factor to be located in nt 1648 to 1671 of HBV DNA (Fig. 7). This binding activity was also found in extracts from Hep3B and rat liver cells but not in extracts from HeLa cells when the same concentration of extracts was used (data not shown). A similar protein-binding site had also been found by Karpen et al. (5) and was previously considered to be the core promoter element. However, the core promoter, as identified by Honigwachs et al. (4) in the *Stu*I-*Fsp*I (1705 to 1805) 100-bp fragment, could function without this binding site. We proposed that this protein-binding site might be closely related to the new enhancer element. The function of this binding site remains to be elucidated.

How this new enhancer element ENII regulates the HBV

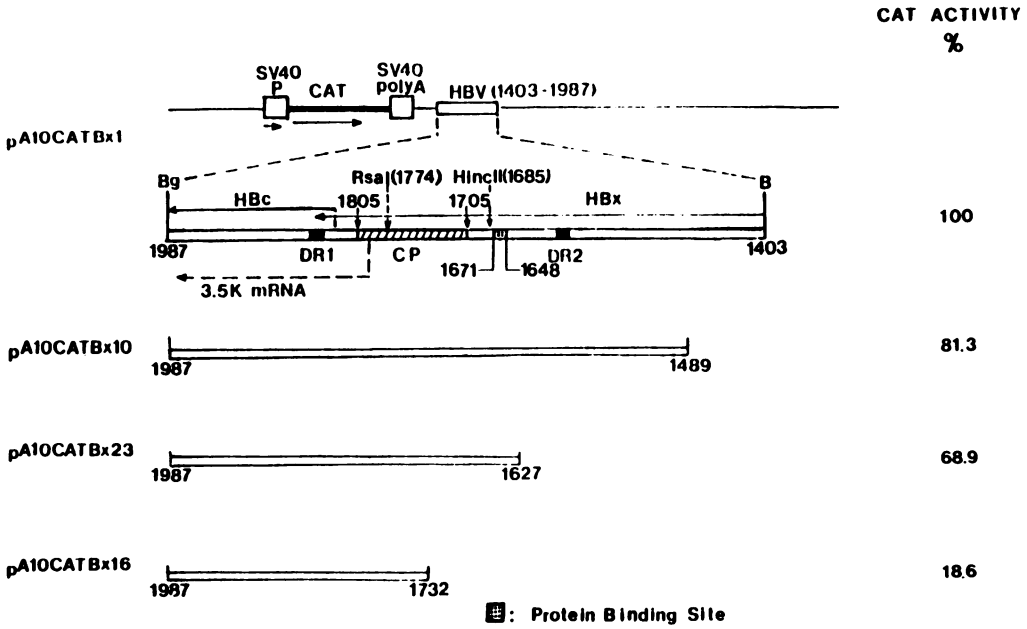



FIG. 6. Deletion analysis of the enhancer ENII-harboring DNA fragment. Plasmid pA10CATBx1 was cleaved at the unique *Bam*HI site, followed by digestion with BAL 31. The digested DNA was pooled and circularized with a *Bam*HI linker. The selected deletion mutants were characterized and sequenced by the dideoxy method. CAT assays were performed as described in the legend to Fig. 1, and activity was expressed relative to the activity of pA10CATBx1, taken as 100.

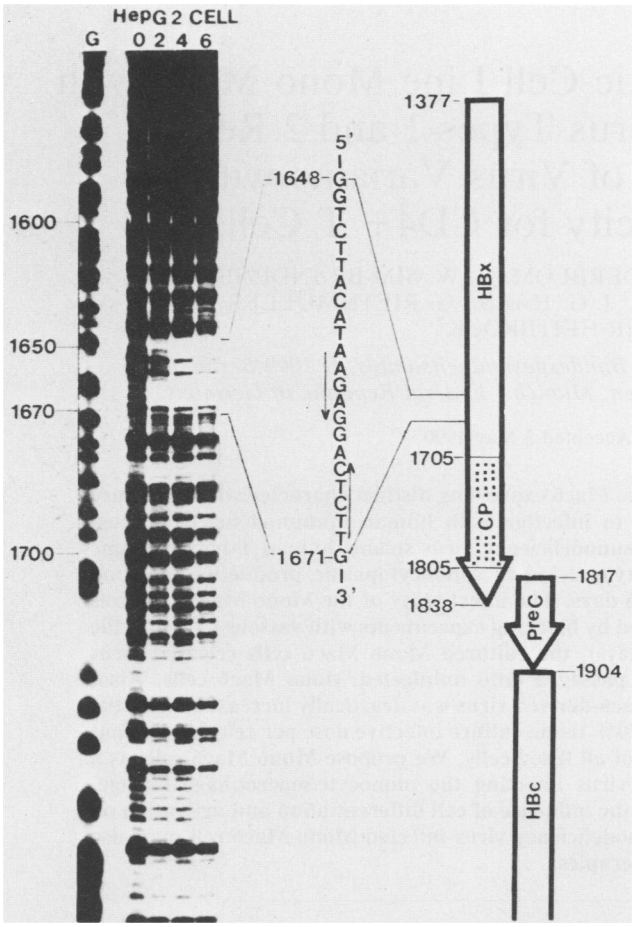



FIG. 7. DNase I footprinting analysis for the identification of a specific protein-binding site. Footprinting was performed by the method of Heberlein et al. (3). HBV DNA fragment *Bam*HI-*Rsa*I (1403 to 1774)
was end labeled at the *Rsa*I site and incubated in the
presence of 5 μg of poly(dI-dC) with nuclear extract of HepG2. As indicated above the lanes, 0, 2, 4, or 6 μg of HepG2 nuclear extract was added. A DNase I concentration of 3 μg/ml was used in each case. Lane G, Sequence ladder. The protein-binding sequence is indicated, and the palindromic sequence is marked by arrows.

promoters and how it interacts with other regulatory elements in HBV genome are now under investigation.

This research was supported by the Chinese Academy of Sciences.

We thank R. Koshy for critical reading of the manuscript and helpful discussions, and we thank X. M. Yu for his kind help.

## ADDENDUM

After we completed this work, J. K. Yee reported similar results (16). He found that an 88-bp *Hin*cII-*Rsa* I fragment (1685 to 1774) of HBV contains a functional promoter and a strong liver-specific enhancer. His findings are in good agreement with our results.

## LITERATURE CITED

1. **Gan, R. B., M. J. Chu, L. P. Shen, S. W. Qian, and Z. P. Li.** 1987. The complete nucleotide sequence of the cloned DNA of hepatitis B virus subtype adr in pADR-1. Sci. Sin. Ser. B **30**:507–521.
2. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
3. **Heberlein, U., B. England, and R. Tjian.** 1985. Characterization of *Drosophila* transcription factors that activate the tandem promoters of the alcohol dehydrogenase gene. Cell **41**:965–977.
4. **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.
5. **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.
6. **Shaul, Y., W. J. Rutter, and O. Laub.** 1985. A human hepatitis B virus enhancer element. EMBO J. **4**:427–430.
7. **Siddiqui, A., S. Jameel, and J. Mapoles.** 1986. Transcriptional control elements of hepatitis B surface antigen gene. Proc. Natl. Acad. Sci. USA **83**:566–570.
8. **Spandau, D. F., and C. H. Lee.** 1988. *trans*-Activation of viral enhancers by the hepatitis B virus X protein. J. Virol. **62**: 427–434.
9. **Sureau, C., J. L. Romet-Lemonne, J. I. Mullins, and M. Essex.** 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell **47**:37–47.
10. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.
11. **Tognoni, A., R. Catteneo, 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.
12. **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.
13. **Tur-Kaspa, R., Y. Shaul, D. D. Moore, R. D. Burk, S. Okret, L. Poellinger, and D. A. Shafritz.** 1988. The glucocorticoid receptor recognizes a specific nucleotide sequence in hepatitis B virus DNA causing increased activity of the HBV enhancer. Virology **167**:630–633.
14. **Vannice, J. L., and A. D. Levinson.** 1988. Properties of the human hepatitis B virus enhancer: position and cell type nonspecificity. J. Virol. **62**:1305–1313.
15. **Wu, X. F., Y. Z. Zhou, Z. M. Feng, Z. P. Li, and S. Y. Xia.** 1983. Cloning and restriction mapping of human HBV genome serotype adr. Sci. Sin. Ser. B (Engl. ed.) **26**:954–960.
16. **Yee, J. K.** 1989. A live-specific enhancer in the core promoter region of human hepatitis B virus. Science **246**:658–661.
17. **Zahm, P., P. H. Hofschneider, and R. Koshy.** 1988. The HBV X-ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. Oncogene **3**:169–177.