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

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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 BamHI 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 BstEII site upstream of the pre-S1 region of the S gene followed by insertion of a BamHI linker, a new BamHI site was introduced in place of the BstEII site. The resulting plasmid, pADR-2B, has two BamHI sites. HBV DNA of pADR-2B was divided into two parts by BamHI digestion. One fragment of 1.8 kilobase pairs (kb) contains the pre-S1-pre-S2-S coding sequence, the SPI promoter, the glucocorticoid receptor-binding 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 transflect 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 the 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 BglII into three fragments. The BamHI-BglII 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 BglII-BglII fragment (1988 to 2426) contains the middle portion of the core antigen-coding sequence. The BglII-BamHI (BstEII) fragment (2426 to 2818) contains the C terminus of the core antigen and the promoter SPI sequence of the pre-S1 gene (Fig. 3). Three of these fragments were inserted into the BamHI site of pA10CAT, producing pA10CATB1x3, pA10CATB1x2, and pA10CATB1x1, respectively. Upon transfection of HepG2 cells, only pA10CATB1x1, which contains the 584-bp BamHI-BglII fragment, could stimulate SV40 promoter and enhanced CAT activity (Fig. 4). Furthermore, the stimulatory activity was similar when this fragment was inserted into the BglII site of pA10CAT upstream of the SV40 early promoter in different orientations, as shown in Fig. 4 for pA10CATB2x3 and pA10CATB3x2. As mentioned, the BamHI-BglII (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 BamHI-BglII (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 BamHI-BglII 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...
FIG. 1. Construction of CAT plasmids pA10CATB1.4 and pA10CATB1.8. In pADR-2B, a BamHI site was introduced in place of the BstEII 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.

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.
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 pAl0CATBx1 from the BamHI site (Fig. 6). It was found that deletion of up to 224 bp of BamHI-BglII fragment from the BamHI 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. 3. HBV genome structure between Rsal and BstEII (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 BstEII site has been changed to BamHI 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 EcoRI as nt 1, since there is no EcoRI site within HBV DNA of pADR-1 (1, 14).](http://jvi.asm.org/)

![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 BamHI site or the upstream BglII site of the CAT expression unit of pAl0CAT. The BamHI-BamHI (BstEII) 1.4-kb fragment in pAl0CATB1.4 was the HBV DNA from nt 1403 to 2818. The BamHI-BglII HBx fragment in pAl0CATBx1, pA10CATBx2, and pA10CATBx3 was the HBV DNA from nt 1403 to 1987. The BglII-BglII HBc fragment in pA10CATBc was the HBV DNA from nt 1988 to 2426. The BglII-BamHI (BstEII) fragment in pA10CATBr was the HBV DNA from nt 2427 to 2818. CAT activity was determined by densitometric scanning of autoradiographs and expressed relative to the activity of pSV2CAT, taken as 100. Values are means of three independent experiments.](http://jvi.asm.org/)
the synthesis of a truncated X gene product, a cotransfection experiment was performed. The BamHI-Stul (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 pAlOCAT. 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 BamHI-BglII fragment (1403 to 1987) and a shorter fragment BamHI-

HincII (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 Stul-FspI (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...
promoters and how it interacts with other regulatory elements in HBV genome are now under investigation.

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ADDENDUM

After we completed this work, J. K. Yee reported similar results (16). He found that an 88-bp HincII-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