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The Genome of Hepatitis B Virus Contains a Second Enhancer: Cooperation of Two Elements within This Enhancer Is Required for Its Function

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Previous studies have identified an enhancer (enhancer I) at nucleotides (nt) 1074 to 1234 in the genome of the human hepatitis B virus (HBV), which locates immediately upstream from the X gene. By analysis of the expression of the chloramphenicol acetyltransferase gene driven by a heterologous simian virus 40 early promoter, we describe the identification of a second enhancer (enhancer II) at nt 1636 to 1741, which locates downstream of enhancer I and immediately upstream of the core gene. With various deletions at the 5′ end of enhancer II, a positive regulatory element was identified at nt 1636 to 1690 (the II-A element), with the 5′ boundary between nt 1636 and 1671. The II-A element alone did not have an enhancer function, but the enhancer activity was achieved by the concomitant presence of the sequence from nt 1704 to 1741 (the II-B element). The II-B element alone did not have enhancer activity. These results indicate that cooperation between the II-A and II-B elements is required to exhibit the enhancer activity of enhancer II. We also show that enhancer II stimulates the transcriptional activity of both the SPI and SPII promoters of the surface gene. Therefore, the SPI promoter activity is regulated by the proximal HNF-1 binding element and the distal enhancers I and II. These results indicate that multiple regulatory elements scattered over the whole viral genome are involved in the regulation of expression of each individual HBV gene and that the same regulatory element controls the expression of different HBV genes. The relative positions of these regulatory elements in the HBV genome suggest that they may control the expression of HBV genes in a coordinate and cooperative manner.

Hepatitis B virus (HBV) is a small DNA virus with a partially single-stranded 3.2-kilobase (kb) genome. The virion has a diameter of approximately 42 nm, with a 27-nm core. The outer envelope of the virion is arrayed by three surface (S) proteins—the major S, the middle S, and the large S (6, 22, 35, 44, 47). The virus is responsible for causing acute and chronic hepatitis, and chronic carriers of HBV are at a highly increased risk of developing hepatocellular carcinoma (5, 46). Although the viral genome and RNA transcripts can be detected in extrahepatic tissues of HBV-infected chimpanzees and in transgenic mice carrying the HBV DNA (4, 8, 14, 15), liver is still the principal site of clinical disease in which HBV actively replicates.

In the productive infection, the major transcripts are the 3.5- and the 2.1-kb RNAs (8). The 3.5-kb RNA acts as the template for reverse transcription of the HBV genome and is also used for the synthesis of the core protein as well as the viral DNA polymerase (40, 45). The 2.1-kb RNA which is used for the synthesis of both the middle and the major surface proteins is transcribed from the proximal simian virus 40 (SV40)-like promoter (SPII). A minor 2.4-kb transcript for the synthesis of the large S protein is transcribed from the distal TATA-like promoter (SPI) (7, 28, 36).

We have previously demonstrated that both the SPI and the SPII promoters display a preference for differentiated hepatoma cell lines (11). The liver- and differentiated state-specific transcriptional activities of the SPI promoter are controlled by the combined action of a HNF-1 binding element, lying between 68 and 95 base pairs (bp) upstream of the RNA cap site in the SPI promoter region, and the HBV enhancer, which is located downstream of the coding sequence of the S gene (11, 12). The liver- and differentiated state-specific transcriptional activities of the SPII promoter are contributed mainly by the upstream flanking sequence in the promoter region (11, 13, 37). In this report, we describe the identification of a second enhancer sequence (enhancer II) in the HBV genome; enhancer II is situated downstream of the previously identified enhancer (enhancer I) (10, 43). Enhancer II activates the transcriptional activity of both the SPI and SPII promoters in a liver- and differentiated state-specific manner. Furthermore, enhancer II was found to be composed of two elements, II-A and II-B, which cooperation is essential for the activating function of enhancer II.

MATERIALS AND METHODS

Plasmid constructions. The pGEM3ZF(−)-based plasmid pA3SpHBs2775 (Fig. 1A) contains the 2,775-bp sequence corresponding to map positions at nucleotides (nt) 2432 to 1990 of HBV (*adw* subtype, with the *Eco*RI site numbered 1). A head-to-tail tandem repeat trimer (A3) of a 237-bp *Bcl*I-*Bam*HI fragment corresponding to the polyadenylation signal of SV40 was inserted immediately upstream of the surface gene to block the nonspecific upstream transcription. Plasmid pA3SpHBs2025 (Fig. 1A) contains the sequence corresponding to nt 2432 to 1234. Sequences of HBV corresponding to nt 1852 to 1990 (a sequence that contains the polyadenylation signal sequence of HBV), nt 1805 to 1990, and nt 1403 to 1990 were inserted at nt 1234 of pA3SpHBs2025 in a sense orientation to generate a series of plasmids, pA3SpHBs2025/1852-1990, pA3SpHBs2025/1805-1990, and pA3SpHBs2025/1403-1990, as shown in Fig. 1A.

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The plasmid pA3SVpCAT (Fig. 2B) contains the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the SV40 early promoter and the polyadenylation signal sequence of SV40 [SV40 poly(A)] located downstream of the CAT gene. As shown in Fig. 2A and 5A, insertion of the HBV sequences from nt 1403 to 1990, nt 1466 to 1990, nt 1636 to 1804, nt 1636 to 1741, nt 1636 to 1703, nt 1704 to 1741, nt 1636 to 1851, nt 1672 to 1851, nt 1687 to 1851, nt 1704 to 1851, nt 1636 to 1690, nt 1636 to 1690, together with nt 1704 to 1741 downstream from SV40 poly(A) in the sense orientation generates a series of plasmids. As shown in Fig. 3A, the 106-bp fragment from nt 1636 to 1741 was inserted downstream from SV40 poly(A) in the antisense orientation and in the upstream of the SV40 early promoter in either sense or antisense orientation to generate pA3SVpCATENII, pA3ENIISVpCAT, and pA3ENIISVpCAT.

A 711-bp fragment containing three tandem repeats of SV40 poly(A) was inserted upstream of the SPI promoter in p(−95)SpICAT, which contains the upstream 95-bp (nt 2717 to 2828) sequence of the transcriptional start site (12), to generate pA3 (−95)SpICAT. The same 711-bp fragment was inserted upstream of the SPII promoter in pA3(−277) SpIICAT, which contains 277 bp (nt 2914 to 25) of the transcriptional start site, to generate pA3(−277)SpIICAT. The sequence from nt 1636 to 1804 containing the enhancer II was inserted downstream of the CAT gene in pA3(−95) SpICAT and pA3(−277)SpIICAT to generate enhancer II-containing plasmids pA3(−95)SpICATENII and pA3(−277) SpIICATENII.

Cell lines, transfections, and CAT assays. The three human hepatoma cell lines, HuH-7 (31), HepG2 (1), and HA22T/VGH (9), as well as a human cervical carcinoma cell line (HeLa), were cultured in Dulbecco modified Eagle medium (Flow Laboratories, North Ryde, Australia) supplemented with 10% fetal calf serum (Boehringer Biochemical, Mannheim, Federal Republic of Germany), 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere. Cells were transfected with plasmids containing the CAT or surface gene by the calcium phosphate precipitation method (21).

CAT assays were performed by the method of Gorman et al. (21) with a slight modification as described previously (11). The CAT activity was normalized with the value of CAT activity of pSV2CAT. Where CAT activity was high, the cell lysate was diluted in a series to assay its CAT activity.

S1 nuclease protection assay. Plasmids containing the surface gene were cotransfected with plasmid pSV2CAT into HuH-7 cells. Twenty-four hours after transfection, total cellular RNA was isolated from transfected cells by the guanidinium-cesium chloride method. Probes (as shown in Fig. 1C) used for the S1 nuclease protection assay for the surface transcripts and for the CAT transcript were a *Sma*I-*Xba*I fragment excised from plasmid pA3SpHBs2775 and a *Bam*HI-*Eco*RI fragment excised from plasmid pA3(−95) SpICAT, respectively. Briefly, pA3SpHBs2775 was first digested with *Xba*I (at nt 250 of HBV), dephosphorylated with calf intestine alkaline phosphatase, labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and chased. After digestion with *Sma*I, the 1,039-bp fragment was then isolated and ethanol precipitated. The probe for detecting the CAT transcript was prepared by the same procedure, except that pA3(−95)SpICAT was first digested with *Eco*RI followed by *Bam*HI, and the 370-bp fragment was isolated. Twenty micrograms of total RNA was mixed with 10^5 cpm of probe in 20 μl of hybridization buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA, 0.4 M NaCl, 80% formamide}, denatured at 85°C for 10 min, and hybridized at 46°C for 10 h. For S1 nuclease digestion, 12.5 U of S1 nuclease (Bethesda Research Laboratories, Gaithersburg, Md.) were used to digest the RNA-DNA hybrids in 180 μl of S1 digestion buffer (0.28 M NaCl, 50 mM sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$, 29 μg of denatured salmon sperm DNA) at 37°C for 1 h. The ethanol-precipitated DNA fragments were analyzed in a 4% urea-polyacrylamide gel, with 10^4 cpm of end-labeled *Sau*96I fragments of pBR322 as size markers.

RESULTS

The sequence downstream of the HBV enhancer increases the level of the surface gene transcripts. Plasmid pA3SpHBs2775 (Fig. 1A) contains the whole surface gene of HBV from nt 2432 to 1990, including the HNF-1 binding element (nt 2719 to 2744), the TATA box of the SPI promoter (nt 2784 to 2790), the SPII promoter (nt 3012 to 30), the enhancer (nt 1074 to 1234), and the polyadenylation signal sequence. After transfection into differentiated hepatoma HuH-7 cells, total RNA was isolated. By using the S1 nuclease protection assay, a single transcript initiated from the SPI promoter at nt 2812 and three transcripts initiated from the SPII promoter at nt 3196, 3215, and 6 were detected as described by Yaginuma et al. (51). The plasmid pA3SpHBs2025/1852-1990 (Fig. 1A) has a deletion from nt 1235 or 1851, but the surface transcripts still use their own polyadenylation signal sequences. As shown in Fig. 1B, the deletion led to a dramatic reduction in the level of all surface transcripts. The plasmid pA3SpHBs2775 has a potential to encode the X protein, whereas pA3SpHBs2025/1852-1990 does not. Whether the X protein plays a role in the regulation was determined by cotransfection of an X expression plasmid with pA3SpHBs2025/1852-1990. The result indicates that the effect of X protein is very limited (not shown).

The HBV sequence containing nt 1805 to 1990 or nt 1403 to 1990 was inserted downstream from nt 1234 of pA3SpHBs2025. As shown in Fig. 1B, the sequence from nt 1403 to 1990 resulted in a significant increase in the level of surface transcripts. The same result was observed in another differentiated hepatoma cell line, HepG2 (not shown). This result is also consistent with the observation that the X protein did not play a significant role in the increase of surface transcripts, because the sequence from nt 1403 to 1990 does not contain the X promoter or the initial 10 amino acids of X protein. This regulatory effect may be explained by the increase of the transcriptional activity of surface gene promoters or the stabilization of the surface transcripts.

The sequence from nt 1636 to 1741 has an enhancer function. To determine whether the nucleotide sequence from nt 1403 to 1990 could activate a heterologous gene promoter, this sequence was inserted downstream from the CAT gene driven by the early promoter of SV40 in the plasmid pA3SVpCAT to generate pA3SVpCAT/1403-1990 (Fig. 2A). Both pA3SVpCAT and pA3SVpCAT/1403-1990 were transiently transfected into HepG2 cells, and the CAT activity was assayed. As shown in Fig. 2, the sequence from nt 1403 to 1990 resulted in a 69-fold increase of the CAT activity. Deletion of the 5′ end to nt 1466 or 1636 and the 3′ end to nt 1804 or 1741 did not affect the stimulating activity. However, dissection of the 106-bp fragment (nt 1636 to 1741) into two subfragments of 68 (nt 1636 to 1703) and 38 (nt 1704 to 1741) bp led to a loss of the stimulating activity. Thus, the

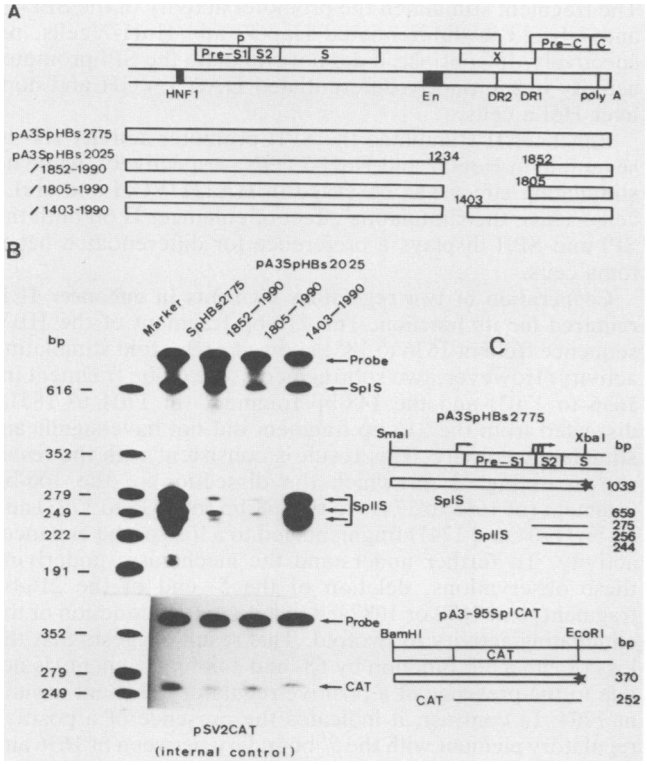

FIG. 1. Increase of the level of HBV surface transcripts by the HBV sequence from nt 1403 to 1851 in HuH-7 cells. (A) A schematic diagram of the HBV sequence (with *adw* subtype and *Eco*RI site numbered 1) in a series of surface gene plasmids. The plasmid pA3SpHBs2775 contains the sequence from nt 2432 to 1990. A series of pA3SpHBs2025 plasmids containing the sequence from nt 2432 to 1234 and different fragments corresponding to nt 1852 to 1990, nt 1805 to 1990, and nt 1403 to 1990 was constructed. The gene organization of the whole surface gene from nt 2432 to 1990 is shown at the top. Open boxes represent HBV sequences present in the constructs. The numbers at the ends of the boxes indicate the nucleotide numbers in the HBV genome. These plasmids were transfected individually into HuH-7 cells, and total RNA was isolated 24 h posttransfection. (B) S1 nuclease protection assay by pA3SpHBs2775 (indicated as SpHBs2775) and a series of pA3SpHBs2025 plasmids. Total RNA (20 μg) was used for the assay. The transcripts from the SPI and SPII promoters are indicated as SpIS and SpIIS, respectively. The end-labeled *Sau*961 fragments of pBR322 were used as the size markers (indicated as Marker). (C) Diagram of the probes used in the S1 nuclease assay and the protected fragments detected. A 1,039-bp 5′-end-labeled *Sma*I-*Xba*I fragment of pA3SpHBs2775 and a 370-bp 5′-end-labeled *Eco*RI-*Bam*HI fragment of pA3(−95)SpICAT were probes for surface and CAT transcripts, respectively. The positions of the cap sites of the surface transcripts and the direction of transcription are indicated by arrows. The correctly initiated transcript from the SPI promoter protects a 659-bp fragment, while transcripts initiated from the SPII promoter protect 275-, 256-, and 244-bp fragments. The correctly initiated CAT transcript protects a 252-bp fragment.

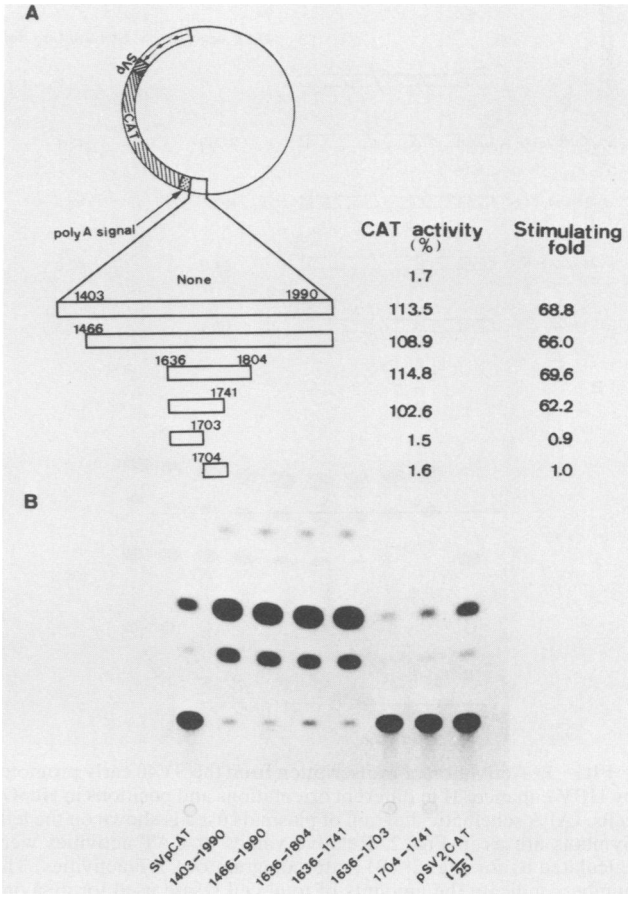

FIG. 2. Activation of the SV40 early gene promoter by HBV enhancer II in HepG2 cells. Different HBV DNA fragments as indicated were inserted downstream from the CAT gene driven by the SV40 early promoter. The plasmid pSV2CAT was used as a control for normalizing CAT activities. Plasmids were transfected into HepG2 cells, and a CAT assay was performed for 1 h at 37°C. (A) Summary of normalized CAT activities. The plasmids containing different fragments of HBV sequences are shown on the left. →→→, Trimer of SV40 poly(A) (shown as poly A signal). SVp, SV40 early promoter. Open boxes represent the HBV sequences, with the numbers indicating the nucleotide numbers in the HBV genome. Each cell lysate was diluted in a series to quantitatively calculate the CAT activities, which were normalized to a percentage of the pSV2CAT activity. The values are the means of four experiments, with a standard deviation of 5%. (B) Autoradiogram of CAT activities. Note that the amount of cell lysate used for pSV2CAT is one twenty-fifth of other samples.

minimal sequence required in conferring the stimulation of a heterologous gene promoter is from nt 1636 to 1741.

Furthermore, the 106-bp fragment of the sequence from nt 1636 to 1741 was placed in both orientations into pA3SVpCAT either immediately upstream from the SV40 promoter (pA3ENIISVpCAT and pA3ENIISVpCAT) or downstream from the CAT gene (pA3SVpCATENII and pA3SVpCATENII) to determine whether the 106-bp fragment has an enhancer function. As shown in Fig. 3, the 106-bp fragment stimulated the CAT activity from 43- to 173-fold in all four constructs. Therefore, the 106-bp fragment of HBV sequence corresponding to nt 1636 to 1741 exhibits an enhancer property. To distinguish this enhancer from the previously characterized enhancer mapping at nt 1074 to 1234 (41, 43), we refer to the enhancer at nt 1074 to 1234 as enhancer I and the enhancer at nt 1636 to 1741 as enhancer II.

Enhancer II displays a specificity for differentiated hepatoma cells. To address the question of tissue specificity of enhancer II, its stimulating activity on the SV40 early promoter was determined in differentiated HepG2 and HuH-7, poorly differentiated HA22T/VGH, and non-liver HeLa cells. The stimulating activities in HepG2 and in HuH-7 cells

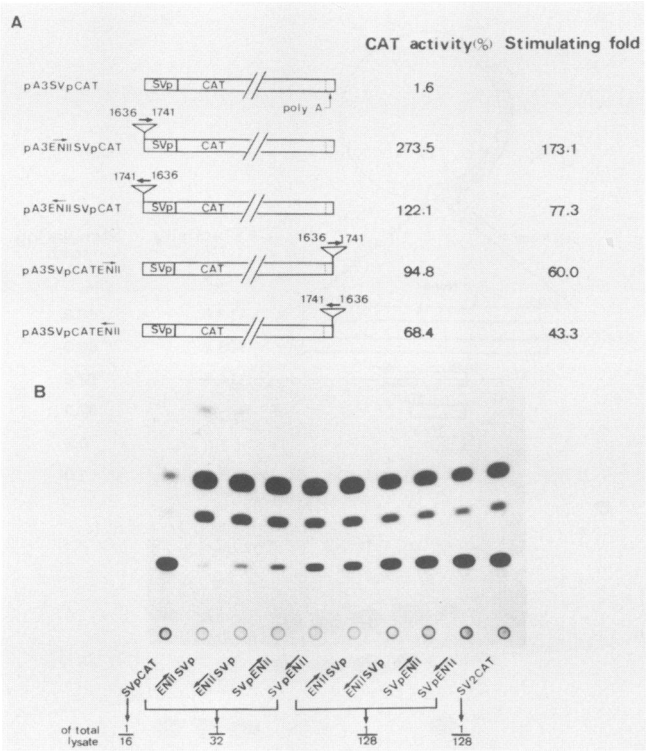

FIG. 3. Activation of transcription from the SV40 early promoter by HBV enhancer II in different orientations and positions in HepG2 cells. (A) A schematic diagram of plasmids used is shown on the left. Symbols are as in Fig. 2. Relative values of CAT activities were calculated as for Fig. 2. (B) Autoradiogram of CAT activities. The numbers indicate the amounts of total cell lysate used for assaying the CAT activity.

were 60- and 10.2-fold, respectively, while no significant stimulation was observed in the poorly differentiated HA22T/VGH and the non-liver HeLa cells (Table 1). Thus, the activating effect of the enhancer II has a preference for differentiated hepatoma cells, especially HepG2 cells.

Enhancer II stimulates the SPI and SPII promoters of the surface gene in a differentiated hepatoma cell-specific manner. We have shown (Fig. 1) that the sequence containing enhancer II increases the level of surface transcripts. To determine whether enhancer II (nt 1636 to 1741) stimulates the promoter activity of SPI and the SPII of HBV, the 106-bp fragment of the enhancer II was inserted downstream from the CAT gene driven by the SPI promoter with the upstream 95 bp of the transcriptional start site in pA3(−95)SpICAT and by the SPII promoter with the 277 bp of the transcriptional start site in pA3(−277)SpIICAT, as shown in Fig. 4.

TABLE 1. Differentiated hepatoma cell specificity of enhancer II

Cell line	CAT activity (%)[a]		Stimulating activity (fold)
	pA3SVpCAT	pA3SVpCATENII	
HepG2	1.6	94.8	60.0
HuH-7	1.7	17.3	10.2
HA22T/VGH	1.1	1.2	1.1
HeLa	1.9	2.1	1.1

[a] The relative CAT activity was calculated as a percentage of that of pSV2CAT. The values are the average of four independent experiments.

The fragment stimulated the promoter activity of the SPI 47- and 7.5-fold in differentiated HepG2 and HuH-7 cells, respectively. In contrast, it did not stimulate the SPI promoter activity in the poorly differentiated HA22T/VGH and non-liver HeLa cells.

Enhancer II stimulated the SPII promoter activity six- to sevenfold in HepG2 and HuH-7 cells, respectively, while no stimulating effect was observed in HA22T/VGH and HeLa cells. Thus, the stimulating effect of enhancer II on both the SPI and SPII displays a preference for differentiated hepatoma cells.

Cooperation of two regulatory elements in enhancer II is required for its function. The 216-bp fragment of the HBV sequence from nt 1636 to 1851 showed a 69.6-fold stimulating activity. However, two subfragments, the 68-bp fragment (nt 1636 to 1703) and the 148-bp fragment (nt 1704 to 1851), dissected from the 216-bp fragment did not have significant stimulating activity. This result is consistent with the result shown in Fig. 2, in which the dissection of the 106-bp fragment (nt 1636 to 1741) to two 68-bp (nt 1636 to 1703) and 38-bp (1704 and 1741) fragments led to a loss of the enhancer activity. To further understand the mechanism underlying these observations, deletion of the 5′ end of the 216-bp fragment to nt 1672 or 1687 led to a dramatic reduction of the stimulating activity to twofold. This result suggests that the loss of enhancer function by 68- and 148-bp fragments is not due to the presence of a positive regulatory element around nt 1704. In contrast, it indicates the presence of a positive regulatory element with the 5′ boundary between nt 1636 and 1671. Furthermore, although the 55-bp fragment (nt 1636 to 1690) had no significant stimulating activity, 30.6- or 30.8-fold enhancer activity was achieved by the concomitant presence of the 148-bp (nt 1704 to 1851) or the 38-bp (nt 1704 to 1741) fragment, respectively. We then designate the 55-bp and 38-bp fragments the II-A and II-B elements, respectively. Therefore, enhancer II is composed of at least two interacting elements, II-A (nt 1636 to 1690) and II-B (nt 1704 to 1741), and their cooperation is required for the activity of enhancer II (Fig. 5).

DISCUSSION

The HBV genome, with a size of 3.2 kb, is very small, and its gene organization is extremely compact, as shown in Fig. 6. An enhancer (enhancer I) mapping at nt 1074 to 1234 of the HBV genome, which is located within the polymerase open reading frame and immediately upstream of the transcriptional start site of the X transcript, has been identified (43). Enhancer I has been shown to stimulate the transcriptional activity of the viral surface, core, and X gene promoters (3, 11, 24). In this report, we identify a second enhancer (enhancer II) in the HBV genome which maps at nt 1636 to 1741 and is located within the X open reading frame and immediately upstream from the transcriptional start site of the core transcripts. We also show that enhancer II stimulates the transcriptional activity of viral surface gene promoters (Fig. 4) as well as the core and X gene promoters (our unpublished observation). We have previously shown that the HNF-1 binding element, which is located proximal to the SPI promoter, regulates SPI promoter activity (12). Therefore, multiple regulatory elements are involved in the regulation of the transcription of each individual HBV gene, and the same regulatory element controls the expression of different HBV genes. Their relative locations in the HBV genome suggests that they may serve to coordinately and cooperatively control the expression of different HBV genes

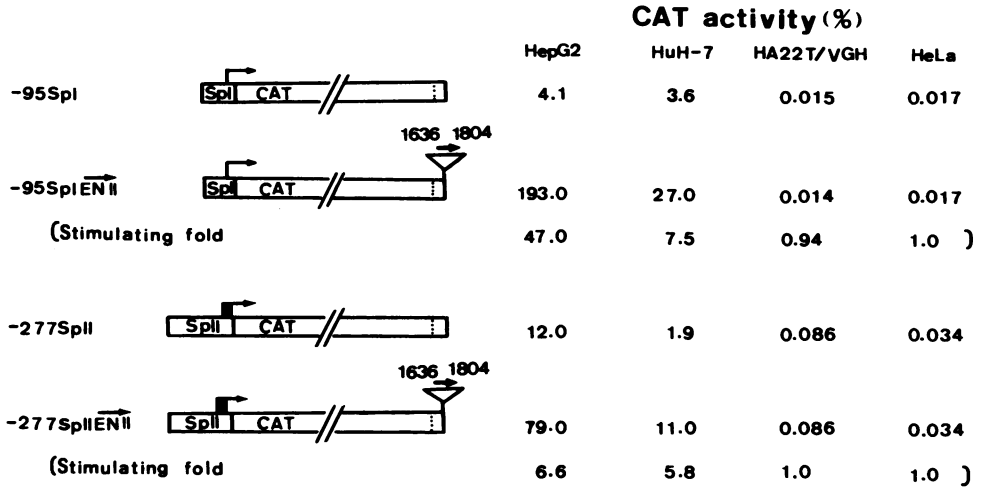

FIG. 4. Activation of the SPI and SPII promoters by HBV enhancer II in HepG2, HuH-7, HA22T/VGH, and HeLa cells. A schematic diagram of plasmids used is shown on the left. The plasmid pA3(−95)SpICAT (−95SpI) contains a 95-bp upstream sequence up to the cap site of the large S transcript (nt 2717 to 2828). The plasmid pA3(−277)SpIICAT (−277SpII) contains the 277-bp (nt 2914 to 25) upstream sequence up to the cap site of the middle and major S transcript. The sequence from nt 1636 to 1804 containing enhancer II (EN II) was placed downstream of the CAT gene. A summary of the normalized CAT activities and levels of stimulating activity (fold) is shown on the right. Parentheses designate the stimulating activity (fold) of enhancer II. Details are as described in the legend of Fig. 2.

to confer the transcriptional program in the viral multiplication cycle.

The 72-bp enhancer of SV40 is composed of several enhansons that act synergistically to stimulate the transcription from an associated promoter (18). For some enhansons, such as GT-1, cooperation with another enhanson is necessary to exhibit an enhancer activity (23, 32–34, 38, 53). Similarly, we show that enhancer II of HBV is composed of two elements, II-A (nt 1636 to 1690) and II-B (nt 1704 to 1741), and that their cooperation is required for the enhancer activity of enhancer II.

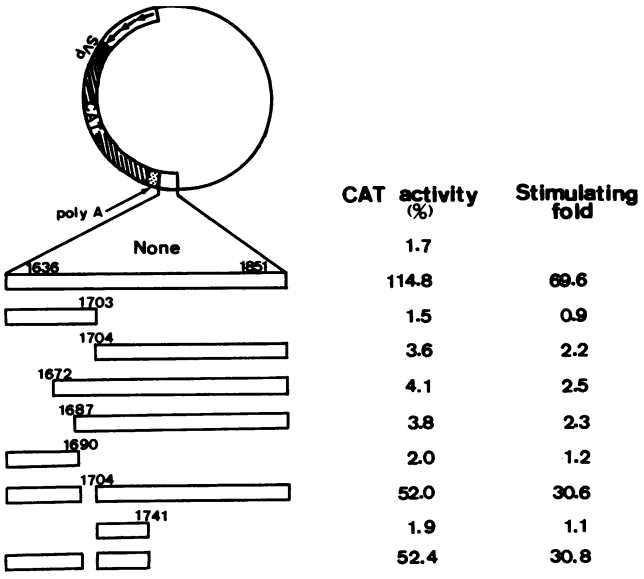

FIG. 5. Cooperation between II-A and II-B elements in the enhancer activity of enhancer II in HepG2 cells. A schematic diagram of different HBV fragments inserted downstream of the CAT gene is shown on the left. A summary of the normalized CAT activities and stimulating activity (fold) is shown on the right. The details of experiments are as described in the legend to Fig. 2.

In the II-A element, the sequence from nt 1641 to 1676 has only one nucleotide different among different subtypes of HBV and is highly conserved in the woodchuck hepatitis virus (19) and the ground squirrel hepatitis virus (20, 39). The sequence 5′-CTTACATAAG-3′ beginning at nt 1651 reveals a weak homology with the consensus sequence 5′-ATTGCG CAAT-3′ of a liver-abundant nuclear factor, C/EBP (2, 25). However, Friedman et al. (17) have recently shown that HepG2 cell contains a very small amount of endogenous C/EBP, and the introduction of exogenous C/EBP into HepG2 by transfection dramatically activates the promoter of the serum albumin gene (17). A 12-nt palindrome of the sequence 5′-AAGAGGACTCTT-3′ beginning at nt 1658 has been shown by Karpen et al. (27) to be protected by a nuclear protein of HepG2. This 12-nt sequence shows a homology with the 5′ upstream region of the mouse and rat apolipoprotein A genes and the rat fatty acid-binding protein gene. In the II-B element, the 19-nt sequence 5′-AAAGAC TGTGTGTTTAAGG-3′ beginning at nt 1712 and protected by the nuclear proteins of HuH-7 has been previously observed by Yaginuma and Koike (50). They have shown that the same protein is present in HepG2. Therefore, whether the C/EBP-homologous, 12-nt palindrome or 19-nt sequence plays an important role in the function of enhancer II needs to be further elucidated. Our observation of the relatively high enhancer activity of the 106-bp fragment (nt 1636 to 1741) in HepG2 compared with that in HuH-7 cells indicates that some of the *trans*-acting factors that interact with different motifs within this 106-bp fragment are probably more abundant in HepG2 than in HuH-7 cells.

Recently, Yee (52) has reported the identification of an 88-bp enhancer in the core promoter region of HBV corresponding to nt 1686 to 1775 of our *adw* sequences. Yee has shown that this 88-bp fragment activates the transcription from the thymidine kinase promoter of herpes simplex virus in a distance- and orientation-independent manner. However, we have shown that the concomitant presence of a positive regulatory II-A (nt 1636 to 1690) with the II-B (nt 1704 to 1741) is necessary for the enhancer activity of enhancer II on the SV40 early promoter. The discrepancy

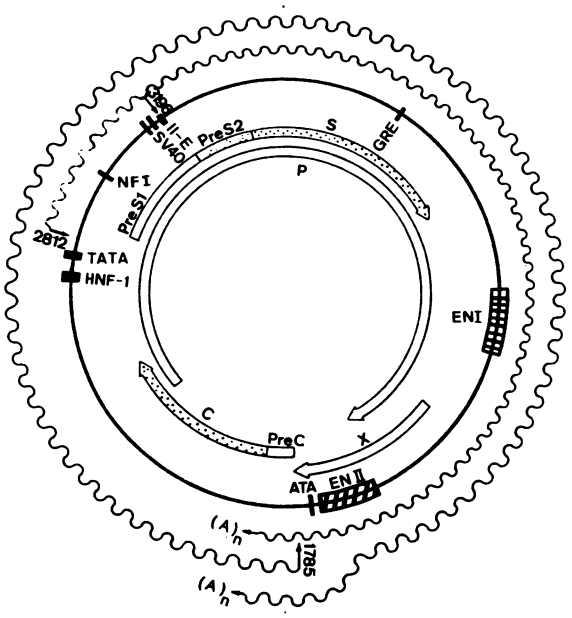

FIG. 6. Map of the putative transcriptional regulatory elements on the HBV genome. The open reading frames are shown on the inside of the circular HBV genome, and the RNA transcripts are shown outside the genome as wavy lines. The positions of putative control elements are shown as solid vertical bars and stippled boxes. HNF-1, HNF-1 binding element located between nt 2719 and 2744 (12). TATA, Position of a TATA-like box at nt 2784 to 2790 which is a component of the SPI promoter. NFI (nt 3107 to 3023) Sequence which interacts with nuclear factor 1 (42). SV40, Regions of sequence homology to the SV40 origin (nt 3103 to 3144) and to the SV40 late promoter (7, 13). II-E (nt 3192 to 3203), Region which shows a positive effect on SPII activity with an enhancerlike function (13). GRE (nt 298 to 369), Region of homology to the consensus sequence for glucocorticoid responsive elements (48, 49). Enhancer I (EN I), at nt 1074 to 1234, interacts with multiple proteins (41, 43). Enhancer II (EN II), at nt 1636 to 1741, is the second HBV enhancer which was identified in this report. ATA (nt 1755-1764), Position of an ATA box which is a presumptive component of the core promoter (6).

may result from the use of different promoters or from a complicated interaction between the enhancer and its neighboring DNA sequences. Further studies will provide a better understanding of the interesting interaction between the promoters and enhancers of this tightly organized genome of HBV.

The *cis*-acting transcription activating elements of HBV, including enhancers I and II and the HNF-I binding element in the HBV genome, are not clustered, but are scattered over the whole genome. The existence of such a scattered multiplicity of transcriptional elements for an individual gene has been shown in many cellular genes. The immunoglobulin light-chain κ gene is regulated by both the κ-intron enhancer and the κ-3′ enhancer (29). Another example is the liver-specific α-fetoprotein gene, which is under the regulation of three distal enhancers located at 2.5, 5.0, and 6.5 kb upstream from the gene and a proximal HNF-1 binding element (16, 20). However, this characteristic of HBV regulatory elements is different from that of small DNA viruses and of retroviruses. For example, in the genome of SV40, multiple transcriptional regulatory elements are clustered in a dimer of the 72-bp enhancer which is positioned between the transcription start sites of both early and late genes in close proximity (26). Although HBV has been suggested to share a common evolutionary origin with retroviruses (30), the transcriptional regulatory elements in retroviruses have so far been mapped very close to the long terminal repeat region. Therefore, the organization of transcriptional activating elements in the HBV genome has a unique feature, which provides an excellent model system to understand the regulation of a viral transcriptional program.

ACKNOWLEDGMENTS

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