Transcriptional Regulation of Precore and Pregenomic RNAs of Hepatitis B Virus

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Hepatitis B virus (HBV) infection, either acute or chronic, has been one of the leading health problems in the world. To understand the HBV life cycle and disease process, we set out to study the regulation of viral gene expression. In this paper, we report the characterization of the HBV core promoter: two 3.5-kb transcripts, precore and pregenomic, are made from it. The latter is itself a template for viral genome replication and also encodes viral proteins essential for both viral replication and virion assembly. We identify a short sequence (from nucleotides [nt] 1744 to 1851, referred to as the basic core promoter [BCP]) that is sufficient to direct correct initiation of both precore and pregenomic messages. In addition, the two appear to be regulated in a coordinate manner. Sequences upstream of the BCP (from nt 1636 to 1744, referred to as the core upstream regulatory sequence [CURS]), have a strong stimulating effect on the BCP. Addition of the CURS to the BCP leads to a dramatic increase in both the transcription of two 3.5-kb messages and the production of 42-nm virions from transiently transfected hepatoma cells. The CURS stimulates the BCP in a position- and orientation-dependent manner. Therefore, it is unlikely that the effect is mediated through enhancer II, which has been localized to the same sequence. Deletion analysis of the CURS suggests that it contains multiple regulatory elements that control the BCP in an interactive manner. In accord with this hypothesis, the CURS is found to be bound with many distinct protein factors in footprinting experiments. Among these elements, box α (from nt 1646 to 1668) and box $\gamma\delta$ (from nt 1671 to 1703) are two regulatory elements which individually stimulate promoter activity more than 100-fold.

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis, and prolonged infection has been associated with cirrhosis and hepatocellular carcinoma (2, 31). HBV is a small DNA virus with a partially double stranded 3.2-kb genome. The viral genome consists of four open reading frames coding for the surface, core, polymerase, and X proteins (3, 13, 32). The viral DNA, upon entry into cells in productive infection, undergoes a repair process and forms the covalently closed circular DNA. Transcription of this DNA produces the longer (precore) and shorter (pregenomic) 3.5-kb RNAs. The production of these RNAs is of special interest in that they have dual functions: the pregenomic RNA is packaged into nucleocapsids along with the viral polymerase and serves as the template for viral genome replication mediated through a mechanism similar to that of the retrovirus, while both of them encode such important viral proteins as core protein, polymerase (by pregenomic RNA), and e antigen (by precore RNA) (9, 26, 29, 34). The regulation of expression of these messages is the pivotal event that governs the viral replication cycle. Studies of this transcription have been facilitated by an in vitro viral production system. Transient transfection of HBV DNA into differentiated human hepatoma cells can initiate both viral transcription and replication that closely resemble what happens in productive infection in vivo (4, 30, 36).

The 5' ends of longer and shorter 3.5-kb RNAs have been mapped by examination of the viral RNA isolated from either infected liver or transiently transfected hepatoma cells. The longer precore RNA initiates at nucleotides (nt) 1785 to 1786 and 1791 to 1793, while the shorter pregenomic RNA starts at nt 1818 to 1820 (34, 36). Both 3.5-kb RNAs

end at the only polyadenylation site in the HBV genome. The close proximity of initiation sites for the precore and pregenomic RNAs raises the interesting possibility that the two are regulated in a coordinate manner.

In this study, we examined in detail the promoter region which controls the expression of precore and pregenomic RNAs. Serial deletion mutants were generated from this region and tested for promoter activity. Both RNAs are found to be affected in the same way in all mutants examined, supporting the idea that they are regulated in a coordinate fashion. We identified a region between nt 1744 and 1851 that alone will ensure the precise initiation of both the precore and pregenomic RNAs. The sequence upstream from nt 1744 stimulated promoter activity. Multiple factors whose interactions exert the activating function were found to bind to this region.

MATERIALS AND METHODS

Plasmid constructions. The HBV sequence (Fig. 1 and 9) used in this study is of the *adw* subtype. Numbering of the HBV sequence begins at the unique *EcoRI* site, which is nt 1. All reporter plasmids used in transfection experiments contain a head-to-tail trimeric tandem repeat, referred to as A3, of a 237-bp *BclI*-*BamHI* fragment from the simian virus 40 (SV40) polyadenylation signal. A3 is placed 5' of the promoter sequence of interest and has been shown to stop transcription read-through from spurious upstream initiation. The parental plasmid, containing A3 on a pGEM backbone, is designated pA3/RidB. Plasmid p(1403-1851) was constructed by insertion of a HBV *BamHI* (nt 1403)-*RsaI* (nt 1851) fragment between the *BamHI* and *HincII* sites of the pA3/RidB vector. The bacterial chloramphenicol acetyltransferase (CAT) gene, which exists as a *PstI* frag-

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FIG. 1. Schematic diagram of HBV genome organization and of the probes used as well as the predicted protection products detected by S1 nuclease mapping. The genome organization of HBV is shown at the top. The open reading frames of pre-S1, pre-S2, surface, precore, core, X, and polymerase are designated PreS1, S2, S, PreC, C, X, and P. Enhancer I (nt 1074 to 1234) and enhancer II (nt 1636 to 1741) are indicated as ENI and ENII, respectively (28, 37). The liver-specific regulatory HNF-1 element (nt 2719 and 2744), ubiquitous Oct-1 element (nt 2754 to 2761), and TATA (nt 2784 to 2790) are components of the SPI promoter (7, 39). NF1, SV40-like, and IIE sequences are located in the SPII promoter (8, 27). The glucocorticoid response element (GRE) is within the S open reading frame (33). The initiation sites of RNAs are indicated by arrows. A diagram of the probes and of the predicted protected fragments for S1 nuclease mapping is shown below. The open boxes marked as *Bam*HI-*Bgl*II, *Bgl*II-*Xba*I, and *Bam*HI-*Bam*HI fragments represent probes used to detect the 3.5-kb core, 2.4- and 2.1-kb surface, and 0.8-kb X transcripts, respectively. The predicted protected fragments are shown as solid lines, with numbers indicating the predicted sizes.

ment, was then inserted at the *Pst*I site of the vector pA3RI/dB and p(1403-1851) to generate pA3-CAT and p(1403-1851)CAT, respectively. Plasmids p(1636-1741)CAT (or pCURS-CAT), p(1636-1851)CAT (or pCURS/BCP-CAT), p(1672-1851)CAT, p(1687-1851)CAT, and p(1704-1851)CAT (or pCURS-B/BCP-CAT) were constructed by replacing the sequence from nt 1403 to 1851 on the p(1403-1851)CAT vector with the HBV sequence from nt 1636 to 1741, 1636 to 1851, 1672 to 1851, 1687 to 1851, and 1704 to 1851, respectively. Plasmids p(1718-1851)CAT, p(1728-1851)CAT, and p(1744-1851)CAT (or pBCP-CAT) were generated by BAL 31 digestion of p(1704-1851)CAT according to the standard protocol, and their sequences were confirmed by double-stranded DNA sequencing (18). Plasmids p α /BCP-CAT and p $\gamma\delta$ /BCP-CAT were constructed by insertion of the oligonucleotide sequences corresponding to box α and box $\gamma\delta$ between the *Bam*HI and *Kpn*I blunt-ended sites immediately upstream to the base core promoter (BCP) of pBCP-CAT. Sequences of oligonucleotides were as follows: for box α , 5'-GATCCATCGATCAAGGTCTTACATAAGAGGACTCTT-3' and 5'-AAGAGTCCTCTTATGTAAGACCTTGATCGATG-3'; for box $\gamma\delta$, 5'-GATCGACTCCCAGCAATGTCAACGACCGACCTTGAGGC-3' and 5'-GCCTCAAGGTGCGTTCGTTGACATTGCTGGGAGTC-3'.

To construct pBCP-CAT vectors with the core upstream regulatory sequence (CURS) in both orientations at either

upstream or downstream positions, a *Bam*HI-*Alu*I fragment was isolated from plasmid p(1636-1851) harbored in *Escherichia coli* GM119 (Dam⁻ Dcm⁻). This fragment was then subcloned into either the *Bam*HI-*Kpn*I site (upstream) or the *Bam*HI-*Sph*I site (downstream) in both orientations to generate the plasmids depicted in Fig. 6.

To construct the constructs pHBV3.6, pHBV3.5, and pHBV3.46, three-way ligations were carried out. The *Eco*RI-*Fsp*I fragments, containing the A3 and core promoter sequences, were taken from pertinent sources that encompass sequences from nt 1636 to 1804, 1704 to 1804, or 1744 to 1804. These fragments were then ligated to the 1,441-bp *Fsp*I-*Pst*I fragment from HBV (nt 1805 to 25) in the presence of an *Eco*RI-*Pst*I-cleaved pGEM vector. The resulting plasmids, pA3(1636-25), pA3(1704-25), and pA3(1744-25), were further engineered by addition of a *Pst*I fragment containing the HBV sequence from nt 25 to 1990 isolated from pSpHBs2775 at their unique *Pst*I sites. The final products, pHBV3.6, pHBV3.5, and pHBV3.46, thus consist of more than unit lengths of the HBV genome that include sequence from nt 1636 to 1990, 1704 to 1990, or 1744 to 1990, respectively.

Cell lines, transfections, and CAT assays. The three human hepatoma cell lines HuH-7 (25), HepG2 (1), and HA22T/VGH (5) as well as human cervical carcinoma cell line HeLa were cultured in Dulbecco modified Eagle's medium (Flow

Laboratories, North Ryde, Australia) supplemented with 10% fetal calf serum (Boehringer Biochemical, Mannheim, Germany), 100 IU of penicillin per ml, 100 mg of streptomycin per ml, 1% nonessential amino acids, 25 mg of amphotericin B (Fungizone) per ml, and 2 mM L-glutamine at 37°C in a 5% CO₂ atmosphere. Cells were transfected with plasmids containing the CAT or HBV DNA constructs by the calcium phosphate precipitation method (16). Since optimal expression of transfected plasmids depends on their supercoiled form, all plasmids used in one set of experiments were simultaneously prepared, checked for supercoil form, aliquoted in small amounts, and stored in 70% ethanol. Each aliquot was used only once. Each set of experiments was performed with two different preparations of plasmids and repeated two to three times for each preparation. Furthermore, 5 µg of target DNA and 1 µg of target with 4 µg of carrier DNA, respectively, were used for the transfection for comparison of the results. CAT assays were performed by the method of Gorman et al. (15), with modifications as previously described (6). CAT activity was normalized by using the CAT activity value of pSV₂CAT as 100%. When CAT activity was high, the cell lysate was serially diluted prior to performance of the CAT assay. Furthermore, for different transfection experiments, the CAT activities were proportional to the amount of the input DNA, suggesting that the transfected DNA templates manifest transcription activities that faithfully reflect their promoter strength.

Preparation of nuclear extracts and heparin-agarose fractionation. Nuclear extracts from differentiated human hepatoma cell lines HepG2 and HuH-7 were prepared as previously described (7). Extracts were stored in small aliquots at -70°C after being quickly frozen under liquid nitrogen. The 25-mg crude nuclear extract was fractionated through a 20-ml heparin-agarose column at 4°C essentially as described by Lichtsteiner et al. (22). They were aliquoted, quickly frozen under liquid nitrogen, and kept frozen at -70°C.

DNase I footprinting analyses. The end-labeled probes were generated by fill-in with α-[³²P]dATP (3,000 Ci/mmol; Amersham International, Amersham, England) at either terminus of a *Bam*HI-*Hind*III fragment containing the 589-bp HBV sequence from nt 1402 to 1990. The plasmid carrying this insert was cut with one enzyme, end labeled, cut with the other enzyme, and then subjected to agarose gel electrophoresis. The labeled fragments were eluted from the agarose gel by a Spin-X filtering unit (Costar, Cambridge, Mass.). The DNase I footprinting assay was performed as previously described (7). Briefly, the crude or fractionated nuclear extracts were incubated with a labeled DNA fragment for 30 min at 4°C. After addition of MgCl₂ and CaCl₂, the proper amount of DNase I was added for digestion for 90 s at room temperature. Reaction products were run on 6% polyacrylamide-8 M urea gels.

RNA extraction from transfected cells and S1 nuclease mapping assay. A total of 2 × 10⁶ cells in a 15-cm cultured dish were transfected with a total of 62.5 µg of DNA. Total cellular RNA was isolated from transfected cells by the guanidinium-cesium chloride method (14). Probes used for the S1 nuclease mapping are shown in Fig. 1. S1 nuclease mapping was performed as previously described (37).

Assay for endogenous DNA polymerase activity. To assay for endogenous DNA polymerase activity (20, 21), the culture supernatant of transfected cells was treated with 1% Nonidet P-40 for 1 h at room temperature and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was then centrifuged at 45,000 rpm for 1 h at 4°C, and the pellet was resuspended in TNE buffer (10 mM Tris-Cl [pH 7.5], 50 mM

NaCl, 0.1 mM EDTA). To assay for endogenous polymerase activity, samples were incubated in polymerase assay buffer containing 50 mM Tris-HCl (pH 7.5), 40 mM NH₄Cl, 5 mM MgCl₂, 0.5% Nonidet P-40, 0.3% 2-mercaptoethanol, 66 mM each dTTP, dGTP, and dCTP, and 0.5 mM α-[³²P]dATP (3,000 Ci/mmol) at 37°C for 2 h, followed by the addition of dATP to a final concentration of 66 mM and further incubation for 1.5 h. Exogenous DNA was digested with *Staphylococcus aureus* nuclease (at a final concentration of 15 U/ml) for 60 min at 37°C and subsequently with proteinase K at 0.5 mg/ml in the presence of 1% sodium dodecyl sulfate for 2 h at 37°C. Samples were then extracted several times with equal volumes of a phenol-chloroform mixture and finally extracted with an equal volume of chloroform. tRNA was added to each sample to a final concentration of 1 mg/ml, and the mixture was passed through a Sephadex G-50 column and precipitated with ethanol. The precipitate was dissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), electrophoresed in 1% agarose gel, and autoradiographed.

RESULTS

Functional identification of the core promoter region. The start sites of the precore RNA have been mapped at nt 1785 to 1786 and 1791 to 1793, while those of the pregenomic RNA have been mapped at nt 1818 to 1820 (36). To identify sequences essential for regulation of the expression of these two 3.5-kb RNAs (we designated these sequences the core promoter), three HBV constructs containing more than a unit length of the HBV genome were made (Fig. 2). Each has the same transcribed region for both 3.5-kb RNAs, equal amount of sequences past the polyadenylation site, but different lengths of sequences upstream of the transcription initiation sites. All three constructs, when transfected into the differentiated human hepatoma cell line HuH-7, possess the machinery needed to synthesize equal amounts of necessary components such as surface and X proteins that later are assembled to form the 42-nm virions (see below). These constructs may, however, differ in the ability to make the precore and pregenomic RNAs. The strength of the core promoter in each construct will determine the amounts of the precore and pregenomic RNAs made, which in turn determines the amount of virions produced.

The constructs pHBV3.6, pHBV3.5, and pHBV3.46 contain HBV sequences starting from nt 1636, 1704, and 1744, respectively. These plasmids were transiently transfected into HuH-7 cells. Three days later, the media were collected and assayed for the production of virus particles by measuring the DNA repairing activity. As shown in Fig. 2, there was very low albeit detectable virus production from cells transfected with pHBV3.46 (unfortunately not detectable on Fig. 2). This finding suggests that the 5' sequence up to nt 1744 contains enough promoter activity to allow synthesis of the 3.5-kb pregenomic transcripts and thereafter the production of virions. In comparison, the presence of additional sequences led to an increase in the production of virions: pHBV3.5, which has sequence up to nt 1704, has a modest capability to produce more virions, while pHBV3.6, which has sequence up to nt 1636, makes a much larger amount of virions. Taken together, these observations suggest that both the upstream 40 bp (from nt 1704 to 1743) and 68 bp (from nt 1636 to 1703) can stimulate the transcription of core transcripts. The former has a mild effect, while the latter has a much stronger effect. These sequences are functionally significant in controlling virion production in vitro and are

FIG. 2. Effects of different 5' ends of the core promoter on the production of HBV virions, as determined by DNA repairing activity. (A) Schematic diagram of the plasmids used. Plasmids pHBV3.6, pHBV3.5, and pHBV3.46 contain the HBV sequence starting from nt 1636, 1704, and 1744, respectively. Each has BCP sequence plus different regions of upstream CURS elements. CURS-A, CURS-B, and BCP denote the sequences from nt 1636 to 1703, 1704 to 1743, and 1744 to 1851 of HBV. (B and C) DNA repairing activity. Plasmids pHBV3.6, pHBV3.5, and pHBV3.46 (3.6, 3.5, and 3.46; lanes 1 to 3) were transfected into HuH-7 cells together with pSV₂CAT as an internal control (see below). The medium was collected 3 days after transient transfection. After treatment with 1% Nonidet P-40, viral cores were pelleted and assayed for endogenous DNA polymerase repairing activity. L and NC represent linear and nick circular forms of HBV DNA; lane M represents labeled *Hind*III-digested lambda DNA fragments used as size markers. The gel was exposed to Fuji X-ray film for 8 h (B) and 72 h (C).

very likely important for analogous biological process in vivo.

To study the transcriptional activity of core promoter directly, levels of the two 3.5-kb messages in day 3 transfected HuH-7 cells were examined by S1 nuclease mapping. As shown in Fig. 3, two 3.5-kb RNAs were undetectable in the pHBV3.46 transfectants. They were, however, detectable in the pHBV3.5 transfectants. Moreover, much stronger signals of these two RNAs were seen in the pHBV3.6 transfectants. These results nicely correlate with observations relating to virus production, further substantiating the positive regulatory role of 40-bp and 68-bp upstream sequences on core promoter transcription.

The levels of the 2.4- and 2.1-kb S transcripts and the clustered 0.8-kb X transcripts were about the same in all three transient transfectants. This finding shows that the difference in the 5' end does not affect transcription from

FIG. 3. Effects of different 5' ends of the core promoter on the levels of HBV RNAs, as analyzed by S1 mapping. Total RNA was isolated 3 days after transfection in HuH-7 cells. Eighty micrograms of total RNA was used for the 3.5-kb core transcripts, while 20 μ g was used for 2.4- and 2.1-kb surface, 0.8-kb X, and CAT messages. Arrows indicate the protected bands of specific transcripts. Size markers were labeled *Sau*96I fragments of pBR322; 3.6, 3.5, and 3.46 represent results obtained from transient transfections with plasmids pHBV3.6, pHBV3.5, and pHBV3.46, respectively. The CAT probe is a *Hind*III-*Eco*RI fragment, and the predicted protected band is 252 bp.

either the S or the X promoter, both of which nevertheless are likely to be stimulated by the downstream second enhancer that all three constructs have (Fig. 1). These results lend proof to our assumption that the difference at the 5' end influences only the synthesis of the precore and pregenomic RNAs and that virion production capability reflects core promoter activity directly.

Identification of the CURS and BCP. It is clear from the data presented above that sequences upstream from nt 1744 have a significant stimulating effect on the core promoter. To better define the sequences that are responsible for this stimulating effect, different segments of the core promoter region were placed in front of a promoterless CAT reporter gene and tested for the ability to drive CAT gene expression. After these plasmids were transiently transfected into the differentiated human hepatoma cell line HuH-7, CAT assays were performed to quantitate reporter gene expression. As shown in Fig. 4, not unexpectedly, the sequence from nt 1636 to 1851 (plasmid pCURS/BCP-CAT; construct a in Fig. 4) displays very high CAT activity. Furthermore, this construct produces RNAs that initiate precisely at the expected sites for the precore and pregenomic RNAs, as judged by S1 nuclease mapping analysis (Fig. 5, lane 3). While the construct with sequence from nt 1704 to 1851 (plasmid pCURS-B/BCP-CAT; construct g in Fig. 4) yields moderate CAT activity, the construct containing sequence from nt 1744 to 1851 (plasmid pBCP-CAT; construct c in Fig. 4) has low CAT activity. This CAT activity of pBCP-CAT is significant, since it is 11-fold higher than that of the promoterless CAT gene control (plasmid pA3CAT; construct b in Fig. 4). The CAT RNAs produced by pBCP-CAT could not be detected. To facilitate examination of the CAT transcripts made by pBCP-CAT, the enhancer I sequence (from nt 1074 to 1234), which stimulates the core promoter, was inserted down-

FIG. 4. Identification of the BCP and its upstream regulatory sequence by the CAT reporter gene in HuH-7 cells. (A) Schematic diagram of the plasmids used and normalized CAT activities. Plasmid pCURS/BCP-CAT (a) contains the HBV sequence from nt 1636 to 1851 inserted upstream of the CAT reporter gene. This HBV fragment was divided into CURS (nt 1636 to 1741) and BCP (nt 1744 to 1851) fragments, which were inserted upstream of the CAT gene to generate pCURS-CAT (d) and pBCP-CAT (c), respectively. The CURS sequence was further divided into box α (nt 1646 to 1668), box $\gamma\delta$ (nt 1671 to 1703), and CURS-B (nt 1704 to 1741) fragments, which were placed upstream of the BCP-CAT region to generate p α /BCP-CAT (e), p $\gamma\delta$ /BCP-CAT (f), and pCURS-B/BCP-CAT (g), respectively. CAT activity is normalized to that of pSV₂CAT, which is taken as 100%. Values are the means of six experiments, with a standard deviation of 10%. (B) Representative autoradiogram of CAT activities. In some cases, serial dilutions of cell lysates were performed to ensure that all CAT assays were done in the linear range. The amount of cell lysates used is indicated; other experimental details are as described in Materials and Methods.

stream of the CAT gene. The resulting plasmid, pBCP-CAT/ENI, produces detectable levels of transcripts with initiation sites identical to those of pCURS/BCP-CAT (Fig. 5, lane 2). Therefore, the 5' sequence up to nt 1744 (as in pBCP-CAT) is sufficient to direct precise initiation in the core promoter. It was of interest to determine whether the CURS itself

FIG. 5. Initiation sites of both precore and pregenomic RNAs analyzed by S1 nuclease mapping. Plasmids pCURS/BCP-CAT and pBCP-CAT/ENI (CURS/BCP and BCP/ENI) were transfected into HuH-7 cells. The HBV 2.1-kb RNA driven by its own promoter (SP II) was cotransfected as an internal control. Plasmid pBCP-CAT/ENI is derived from pBCP-CAT by placing a copy of the enhancer I sequence (nt 1074 to 1234) of HBV downstream of the CAT gene. Total RNA was isolated 1 day posttransfection. Eighty and 20 μ g were used for the S1 mapping analysis of CAT and surface RNAs, respectively. Labeled *Sau*96I fragments of pBR322 were used as size markers. The probe and the predicted protected bands are shown at the right.

functions as a promoter. CAT activity driven by the CURS (nt 1636 to 1743) without the BCP (plasmid pCURS-CAT; construct d in Fig. 4) is 150-fold lower than that with the BCP (pCURS/BCP-CAT; construct a), demonstrating that the CURS functions as a regulatory region but not a promoter.

We thereby conclude that the minimal essential sequence for the core promoter lies between nt 1744 and 1851, referred to as the BCP. In addition, the sequence upstream of nt 1744 has a strong stimulating effect on core promoter strength. The sequence between nt 1636 and 1743, designated the CURS, activates the BCP by 1,900-fold in HepG2 and 180-fold in HuH-7 (Table 1). The CURS can be further divided into two subdomains, CURS-A (nt 1636 to 1703) and CURS-B (nt 1704 to 1743). CURS-A by itself exerts a strong positive regulatory effect on the BCP, though to a smaller extent than does the whole CURS region (130-fold in HepG2 and 34-fold in HuH-7).

The ratio of precore and pregenomic RNAs remained almost the same between pHBV3.6 and pHBV3.5 (Fig. 3) and between pCURS/BCP-CAT and pBCP-CAT/ENI (Fig.

TABLE 1. Regulation of the core gene promoter in differentiated human hepatoma cells

Plasmid ^a	Normalized CAT activity (%) ^b		Induction (fold)	
	HepG2	HuH-7	HepG2	HuH-7
pBCP-CAT	0.040	0.084	1	1
pCURS/BCP-CAT	76.8	14.7	1,900	180
pCURS-A/BCP-CAT	5.28	2.85	130	34
pCURS-B/BCP-CAT	0.328	0.465	8.2	5.5

^a BCP represents nt 1744 to 1851 of the HBV sequence. CURS, CURS-A, and CURS-B represent the sequences from nt 1636 to 1743, 1636 to 1703, and 1704 to 1743, respectively, which are located upstream of the BCP in a sense orientation.

^b Calculated as a percentage of the activity of pSV₂CAT. The variations of different experiments were within 10%. Values are averages of five independent experiments.

TABLE 2. Differentiated hepatoma cell specificity of the core gene promoter^a

Cell line	Normalized CAT activity (%)		Induction (fold)
	pBCP-CAT	pCURS/BCP-CAT	
HepG2	0.037	70.9	1,900
HuH-7	0.075	14.0	190
HA22T/VGH	0.168	0.149	0.90
HeLa	0.101	0.725	7.2

^a For details, see footnotes to Table 1.

5). This finding indicates that the precore and pregenomic RNAs are coordinately regulated by different parts of the CURS in a similar manner.

Differentiated hepatoma cell specificity of CURS effect. As stated above, the CURS in its entirety (nt 1636 to 1743) positively modulates BCP activities in both the HepG2 and HuH-7 cell lines, which are known to have many characteristics of differentiated hepatocytes. This strong regulatory effect, however, is not seen in the poorly differentiated hepatoma cells such as HA22T/VGH or nonliver cells such as HeLa (Table 2). The question of what underlies this salient differentiated liver cell specificity remains to be addressed. It is likely that the factor(s) that stimulates the BCP via interaction with the CURS is present in a functional form only in the differentiated liver cell lineages (see below).

Activation of the BCP by the CURS is position and orientation dependent. We have previously reported the identification and characterization of the second enhancer (37). It was shown that enhancer II activates the expression of homologous promoters such as the HBV surface and X promoters or a heterologous promoter such as the SV40 early promoter in a position- and orientation-independent manner (e.g., stimulation of the SV40 early promoter is 170-, 77-, 60-, and 43-fold with respect to upstream and downstream positions in either sense or antisense orientation) (37). Further analysis reveals that the function of enhancer II is strictly dependent on a bipartite structure, a 23-bp box α (nt 1646 to 1668) and a 12-bp box β (nt 1704 to 1715). Interestingly, the two sequence motifs fall in the CURS region (38).

The position of box α within CURS-A and box β within CURS-B raises the interesting possibility that the activation of the BCP by the CURS is actually mediated through the action of enhancer II. However, this was found not to be the case. First, as shown in Fig. 6, when the CURS is placed in both orientations, either upstream or downstream of the BCP, to test for transcription activation, a sense, upstream position produces by far the greatest stimulation of the BCP, more than 21-fold stronger than the stimulation by an upstream, inverted counterpart. The latter, in turn, produces 12- or 90-fold-stronger stimulation than does downstream placement in either sense or antisense orientation in HepG2 cells. Activation of the BCP by the CURS is therefore both position and orientation dependent and is incompatible with the mode of action known for enhancer II. Similar results were obtained for HuH-7 cells. Second, deletion analysis within the CURS region has shown that CURS-A alone (containing box α) will stimulate the BCP by 130-fold in HepG2 cells. Although the whole CURS region has a much stronger positive effect on BCP activity, these results suggest that CURS-A alone can independently elevate BCP activity. The enhancer II function, however, depends on the presence and interaction of both box α and box β sequences.

Enhancer II is rendered functionless when either of them is missing (38).

In sum, the results bring us to the conclusion that activation of the BCP by the CURS is not mediated to a significant extent by the enhancer II function. It is likely that box α and box β sequences and/or their binding factor(s) are involved in a mechanistically different way in the CURS function.

Multiple factors bind to the CURS region. DNase I footprinting analysis was used to examine the binding of cellular factors within the CURS region. A DNA fragment corresponding to nt 1401 to 1990, which contains the CURS region, was labeled on either the coding or noncoding strand and then subjected to DNase I digestion and incubation with nuclear extracts of HepG2 or HuH-7 cells. Several distinct footprints bracketed with hypersensitive sites, regions I to IV (Fig. 7A), are noted in the CURS region (summarized in Fig. 7C and D). However, a distinct binding pattern of nuclear proteins to region I (nt 1640 to 1681) between HepG2 and HuH-7 was observed. When fractionated nuclear extracts of HepG2 were used, proteins present in the 0.4 and 0.5 M NaCl fractions bind to region I (nt 1645 to 1681), while those in the 0.3 M fraction bind to region II (nt 1705 to 1721) (Fig. 7B; summarized in Fig. 7C and D).

Dissection of the CURS region. To more precisely identify the sequence elements within the CURS region that are responsible for activation of the BCP, serial deletions of the CURS from either the 5' end or the 3' end were made, and the deletion mutants were tested for the ability to stimulate the BCP.

As shown in Fig. 8, incremental removal of sequences from the 5' end of the CURS results in a gradual diminution of promoter-stimulating capability. For example, in HepG2 cells, a steady 5- to 10-fold decrease in stimulation is noted from one deletion mutant to the next-longest one between nt 1636 and 1703. A small yet reproducible increase is seen when the deletion is from nt 1704 to 1718. The decrement is resumed upon removal of sequences from nt 1718 to 1743. Similar results were observed in HuH-7 cells. The most likely explanation is that multiple positive regulatory elements are present throughout the CURS region, with at least one negative element whose function is abolished when sequences from nt 1704 to 1718 are deleted. The sequences from nt 1646 to 1668 and from nt 1704 to 1715 have been previously designated box α and box β . We can further designate the sequence between nt 1671 and 1686 as box γ and the sequence from nt 1687 to 1703 as box δ , both of which may represent unitary sequence motifs that positively regulate BCP activity.

Intriguingly, serial deletions starting from the 3' end of the CURS yield a set of deletion mutants that manifest incongruous changes in activities. Most notably, deletion mutants that have as much sequence as from nt 1636 to 1703 has the same activity as the mutant that contains only nt 1636 to 1668. Deletion mutants that have intermediate amounts of sequences display similar activities. This finding is in sharp contrast to the results for the 5' deletion series in that a 60-fold drop in activity is observed when sequences between nt 1671 and 1703 are omitted.

Moreover, the sequence between nt 1671 and 1703 (box $\gamma\delta$) is not without function by itself: when placed immediately upstream of the BCP, it can exert potent positive regulation (i.e., 170-fold activation). A second discrepancy is observed when box α is placed adjacent to the BCP. Although the exclusion of box α in the 5' deletion series (compare deletion mutant 1671 with the wild type) causes only a minor (fourfold) decrease in stimulation activity, box

FIG. 6. Position- and orientation-dependent activation of the BCP by the CURS in HepG2 and HuH-7 cells. (A) Schematic diagram of the plasmids used, normalized CAT activities, and fold induction. Plasmid pBCP-CAT (a) contained the HBV sequence from nt 1744 to 1851 inserted upstream of the CAT reporter gene. The upstream 106-bp CURS element (nt 1636 to 1741) is placed in both orientations either upstream (pCURS/BCP-CAT [b] and pCURS/BCP-CAT [c]) or downstream (pBCP-CAT/CURS [d] and pBCP-CAT/CURS [e]) of the BCP-CAT region. CAT activity is normalized to that of pSV₂CAT, which is taken as 100%. These plasmids were transfected into HepG2 and HuH-7 cells, and the CAT assay was performed for 1 h at 37°C. Values are the means of six experiments, with a standard deviation of 10%. (B) Representative autoradiogram of CAT activities. The amount of cell lysates used is indicated; other experimental details are as described in the legend to Fig. 4.

α can independently enhance BCP activity by 120-fold. This deletion analysis supports the notion that multiple elements that control BCP activity in both positive and negative fashions are situated within the CURS region. The mode of action for each sequence motif, however, seems to be quite complicated and various.

DISCUSSION

The production of the two 3.5-kb messages (the precore and, especially, pregenomic RNAs) is considered one of the most important events in the HBV replication cycle. Though the precore RNA encodes e antigen, for which no known

function has been described, the pregenomic RNA has a dual role. It codes for polymerase and core protein and itself serves as the template for viral genome replication. Previous studies have mapped the transcription initiation sites for the precore RNA at the nt 1785 to 1786 as well as 1791 to 1793 and those for pregenomic RNA at the nt 1818 to 1820 (36). Other reports have indicated that the sequence from nt 1704 to 1805 displays transcriptional activity when placed upstream of the reporter gene (19, 35). López-Cabrera et al. have reported that the 232-bp sequence from nt 1591 to 1822 contains all components necessary for core promoter activity (23). However, the sequence elements that control the precise initiation of these two messages or regulate tran-

FIG. 7. DNase I footprinting analysis of the core promoter region of HBV. The footprinting analysis was performed by using a *Bam*HI-*Hind*III fragment containing nt 1402 to 1990 of HBV. Either the coding or noncoding strand was end labeled with the Klenow fragment of *E. coli* DNA polymerase. The crude and partially purified nuclear extracts were prepared as described in Materials and Methods. DNase I cleavage sites on naked DNA as well as DNA preincubated with nuclear extracts are shown side by side. The protected regions are indicated by lines on the right, with numbers referring to positions within the HBV genome. Arrows indicate the DNase I-hypersensitive sites. (A) DNase I footprints with crude nuclear extracts of HepG2 and HuH-7 cells on the coding (lanes 1 to 4) and noncoding (lanes 5 to 7) strands. Lanes: 1, G+A sequence marker; 2 and 5, no protein; 3 and 6, 120 μ g of protein from crude nuclear extract of HuH-7; 4 and 7, 120 μ g of protein from crude nuclear extract of HepG2. Lines at the right of lanes 3 and 6 indicate the protected region of HuH-7; lines with numbers at the right of lanes 4 and 7 indicate the protected regions of HepG2. (B) DNase I footprint with crude and fractionated nuclear extracts on the coding strand. Lanes: 1 and 2, G and G+A sequence markers; 3, no protein; 4, 120 μ g of crude nuclear extracts; 5 to 10, 40 μ g each of 0, 0.2, 0.3, 0.4, 0.5, and 1 M NaCl fractions of the heparin-agarose column. Lines at the right of lanes 4 to 9 indicate the protected regions of salt eluents, while lines with numbers at the right of lane 10 indicate the protected regions of crude nuclear extract. (C and D) Summary of the protected sequences in the core promoter region. (C) Protected regions of crude nuclear extracts of HuH-7 (thin lines) and HepG2 (thick lines) on both coding and noncoding strands (only the coding-strand sequence is displayed). Arrowheads indicate the DNase I-hypersensitive sites. (D) Protected regions of crude and fractionated (as indicated) nuclear extracts of HepG2 on the coding strand.

FIG. 9. Map of the transcriptional regulatory elements on the HBV genome. The open reading frames are shown inside the circular HBV genome. The sequence from nt 1636 to 1851 corresponding to enhancer II and the core promoter is expanded on the bottom to indicate relative positions. The core promoter has two components: BCP (nt 1744 to 1851) and CURS (1636 to 1741). The positions of putative box α (nt 1646 to 1668), box γ (nt 1671 to 1686), box δ (nt 1687 to 1703), and box β (nt 1704 to 1715) are shown as open boxes. The positions of other putative control elements are shown as bars and boxes. HNF-1, HNF-1 binding element located between nt 2719 and 2744 (7); TATA, position of a TATA-like box at nt 2784 to 2790, which is a component of the SPII promoter; NF1 (nt 3107 to 3023), sequence which interacts with nuclear factor 1 (27); SV40, regions of sequence homology to the SV40 origin (nt 3103 to 3144) and to the SV40 late promoter (8). II-E (nt 3192 to 3203), region which shows a positive effect on SPII activity with an enhancerlike function (8); XP (nt 1117 to 1234), region which displays promoter activity to express X transcripts (17); GRE (nt 298 to 369), region of homology to the consensus sequence for glucocorticoid response elements (33). Enhancer I (ENI), at nt 1074 to 1218, interacts with multiple proteins (17, 28).

Thus, we are led to conclude that the effect of the CURS on the BCP is not mediated via enhancer II.

Regulatory elements within the CURS have not been clearly studied in previous reports (23, 35). To define more precisely the sequence elements that are the functional constituents, we have made a series of deletion mutants and assayed for their stimulatory activities. We observed that serial truncations from the 5' end of the CURS (nt 1636) yield mutants that, except for a small increase from nt 1704 to 1718, possess decreasing ability to stimulate the BCP. The simplest model would be that there are multiple regulatory elements, both positive and negative, that are arranged in a linear array over the entire CURS region. These elements regulate BCP activity most likely in an additive or synergistic manner. However, serial deletions from the 3' end of the CURS (nt 1743) produce mutants that exhibit unexpected activities, which are not consistent with the simplest model

described above. One surprising observation is that the sequential removal of sequences from nt 1703 up to 1668 in the 3' deletion series does not exhibit a significant drop in BCP stimulation activity, which would be predicted from the 60-fold decrease in potency when a similar sequence is removed in the 5' deletion series. In addition, deletion of a sequence between nt 1636 to 1670 in the 5' deletion series decreases BCP activation by only 4-fold, while the sole presence of a nearly identical sequence (nt 1636 to 1668) is capable of stimulating BCP activity more than 100-fold. To reconcile this discrepancy, we postulate that the different elements within the CURS may function in an interactive or interdependent style. Omission of some elements may affect the function of others either quantitatively or qualitatively. Another likely scenario would be that the stimulation activity of individual motifs depends on their distance and/or spacing relative to the BCP. A weaker element may be rendered stronger by juggling its position and orientation with respect to the basic transcription machinery.

Taken together, the results of deletion analysis reveal a very intricate pattern of functional organization in the CURS region. Such complexity is reminiscent of other well-studied regulatory elements such as the SV40 enhancer. The 72-bp repeat of the SV40 enhancer consists of several operative units called enhansons. Each of these sequence motifs is bound by cellular factors that modulate viral gene expression in a concerted fashion (12, 24). Two functional constituents are identified in our deletion analysis of the CURS region: box α and box $\gamma\delta$. Curiously, box α has been previously implicated in the enhancer II region. It is not clear whether the same or distinct yet overlapping sequence elements are involved in these two different viral gene regulations, nor has the identity or the mechanism of the transcription factor(s) that mediates these controls been identified. Examination of the box α and box $\gamma\delta$ sequences reveals no striking resemblance to other transcription factor binding sites except for a weak homology to the C/EBP consensus exhibited by box α (see below). Clarification of these issues await more detailed dissection of the whole region.

We have also used footprinting analysis to begin to understand the interaction of different transcription factors with the CURS. Consistent with the presence of multiple regulatory elements, multiple footprints over this region are seen. Our results of footprinting experiments with HuH-7 and HepG2 nuclear extracts are very similar to those for rat liver nuclear extracts. López-Cabrera et al. have shown that the C/EBP protein is able to bind to box α (nt 1645 to 1669) and box $\gamma\delta$ (nt 1676 to 1691) sequences (23). Though C/EBP is an attractive candidate that may mediate the stimulatory effect of the CURS as it does for other liver-specific genes, it is probably not the protein bound to box α in HepG2 cells. The reason is twofold. First, only a trace amount of C/EBP protein is found in HepG2 cells (11). Second, the box α -binding proteins in HepG2 cells have a much stronger affinity for the box α sequence than does the recombinant C/EBP protein (38). We have previously proposed that a distinct protein(s) with a different biochemical nature is more likely to be the binding factor(s).

The insertion of an 8-bp *SacI* linker DNA between nt 1726 and 1727 has previously been shown to cause a fivefold decrease in core promoter activity, which correlates with the identification of the specific binding factor as the sequence from nt 1712 to 1730 (35). We have also observed nuclear proteins binding to the sequence from nt 1707 to 1751. Surprisingly, the positive effect of CURS-B on BCP promoter activity is very low.

The CURS displays a very strong specificity for cell type and differentiation stage. The stimulation effect is seen only in well-differentiated human hepatoma cells such as HepG2 and HuH-7 but not in poorly differentiated hepatoma cells such as HA22T/VGH or nonliver cells such as HeLa. However, almost the entire CURS region is protected by nuclear extracts of both HA22T/VGH and HeLa cells, as shown by the DNase I footprinting assays (data not shown). The protection patterns displayed by nuclear extracts of these two cell lines are very similar to those of differentiated HuH-7 and HepG2 cells. One possible explanation is that the transacting factors are distinct between the former and the latter cells. Alternatively, such factors are present ubiquitously but are made functional only in certain cell lineages through specific modifications.

As mentioned before, the BCP contains the initiation sites for both the longer and shorter 3.5-kb transcripts (the precore and pregenomic RNAs). While the CURS stimulates the BCP, we are interested in whether transcriptions of both messages are modulated in the same way. Transcriptions of both messages were found to be stimulated to the same degree by either the upstream 40 bp (nt 1704 to 1743) or 68 bp (nt 1636 to 1703). These results are consistent with the notion that the two 3.5-kb transcripts are coordinately regulated and the CURS exerts equal effects on both of them. It is perhaps not surprising for a virus to economize its genome organization by placing multiple genes under the control of one mechanism. Earlier surveys done on transgenic mice harboring the HBV transgene found a dissociation between expression of the two 3.5-kb transcripts (10). We do not have a good explanation except that BCP may respond to regulation by other elements differentially.

In conclusion, we reported the identification and characterization of the BCP and CURS. The complex functional organization and involvement of the same sequence motif in both enhancer and CURS actions is particularly interesting. A combined biochemical and genetic approach will be most suitable to gain a deeper insight into not only the molecular biology involved but also the relevant disease events that are set in motion.

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