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Identification of a Promoter Region for 3.6-Kilobase mRNA of Hepatitis B Virus and Specific Cellular Binding Protein

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The promoter region for transcription of the 3.6-kilobase mRNA of hepatitis B virus was identified by the chloramphenicol acetyltransferase assay by using HuH-7 hepatoma cells and was found to function directly in virus production by way of the transient expression system of HBV. The 5′-upstream sequence from nucleotides 1573 to 1657 (the transcription start site) was indispensable for promoter function, while the AT-rich sequence (from nucleotides 1581 to 1604) containing a directly repeated sequence TGTT connecting the same flanking sequence PyAAAGAC (where Py is a pyrimidine) at both sides was an essential element within this promoter region. A specific cellular factor which interacted with the essential element was detected in the HuH-7 cell extract. A similar binding factor was also observed in HepG2 and huH2-2 hepatoma cells. This factor may thus be responsible for regulating 3.6-kilobase mRNA, pregenome RNA transcription, or both.

Infection caused by hepatitis B virus (HBV) causes acute and chronic hepatitis, and chronic HBV infection has been found to be closely related to hepatocellular carcinoma (1). From this, it follows that HBV may possibly be a tumor virus. Until recently, studies on the viral life cycle have been hampered by the lack of an adequate in vitro propagation system. However, in vitro culture systems with human hepatoma cells that are capable of producing HBV particles by DNA transfection have been successfully established by various groups of investigators (4, 22, 24, 25, 28). Significant advances in the study of HBV replication and transcription should thus be possible through analysis by in vitro expression systems.

In the HBV-infected liver, two major transcripts of 3.6- and 2.2-kilobase (kb) mRNAs and one minor transcript of 2.6-kb mRNA have been observed during replication (3, 26). The 3.6-kb mRNA is involved in the expression of several protein products such as core antigen, e antigen, and polymerase. It also performs an important function as a pregenome for virus replication (21, 26). The 2.2-kb mRNA is involved in the production of the pre-S2 and S proteins. The minor 2.6-kb mRNA has been shown to produce the pre-S1 protein (11, 18, 23). In addition, 0.8- to 0.9-kb mRNA has been detected in the in vitro expression system by the DNA transfection of HBV DNA (14). This transcript may be related in some way to the expression of the X gene. Thus, four classes of mRNAs have been identified so far in the process of HBV propagation and are known to use the single polyadenylation signal for their termination. The HBV genome may thus possibly possess at least four promoter regions for transcription.

Two similar major transcripts were also observed in the infection of other hepadnaviruses, the woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV). Analysis of the 5′ ends of the two major transcripts from infected woodchucks and ground squirrels indicated that they were heterogeneous for all transcripts (7, 16). For the two major transcripts of HBV, however, use of the transient expression system of transfected HBV DNA with HuH-7 cells made possible a similar analysis of the heterogeneity of the 5′ ends of two major transcripts, the 3.6- and 2.2-kb mRNAs (28). From the data obtained, the promoters for the major transcriptions of HBV appeared to possess certain unique characteristics. The promoter region for the 2.2-kb mRNA may be homologous to the simian virus 40 late promoter sequence (2). The promoter for the 3.6-kb mRNA, however, still remains to be adequately clarified.

In our previous study on HBV production in the transient expression system, efficient transcription and replication of HBV could be observed only in HuH-7 or HepG2 cells, which are highly differentiated hepatoma cells (28). Thus, a certain cellular factor possibly specific for regulating HBV transcription may be present in liver cells.

In the present study, precise identification was made of the promoter region for the 3.6-kb mRNA transcription of HBV by the chloramphenicol acetyltransferase (CAT) assay by using HuH-7 cells. Deletion analysis indicated that the 5′-flanking sequence from nucleotides (nt) 1573 to 1657 (the transcription start site) is indispensable for transcription and that the AT-rich sequence from nt 1581 to 1604 is an essential element in this promoter region. Also, the promoter region was found to participate directly in virus production by the transient expression system of HBV in HuH-7 cells. A specific cellular factor that bound to the essential element of the promoter was observed in the nuclear extract of HuH-7 cells, indicating that this factor is possibly partially responsible for regulating pregenome RNA transcription.

MATERIALS AND METHODS

Construction of deletion mutants for the core particle production assay and CAT assay. Construction of the template plasmids pHBV-2 and pHBV-2Δ*coh* has been described previously (28). The plasmid pHBV-3 was derived from pHBV-2 by excision of the 0.7-kb *Hin*dIII-*Bam*HI fragment and the subsequent insertion of *Hin*dIII linker DNA. To construct deletion mutants of the upstream region of the 3.6-kb mRNA transcript, pHBV-3 was linearized with *Hin*dIII and treated with the exonuclease *Bal* 31. The deleted DNA templates were recircularized by insertion of a *Hin*dIII linker and were used to transform *Escherichia coli* HB101. The extent of deletion in each mutant plasmid was assessed by DNA sequencing (15). The pHBV-4 plasmid was made by excision of the 0.3-kb *Hin*dIII-*Dra*I fragment from pHBV-3 DNA and the subsequent insertion of *Hin*dIII linker DNA.

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The pHBV-2Δ*coh-d*6 plasmid was constructed by treating *Sac*I-linearized pHBV-2Δ*coh* DNA with *Bal* 31 followed by the insertion of *Sac*I linker DNA. The pHBV-3Δ*coh* and pHBV-3Δ*coh-d*6 templates were constructed by replacing 2.0-kb *Bam*HI-*Xba*I fragments with relevant fragments of pHBV-2Δ*coh* and pHBV-2Δ*coh-d*6.

To prepare the plasmids for the CAT assay, the derivative plasmid of pSV2CAT, termed pCAT, was constructed by changing the sites of *Hin*dIII and *Acc*I to *Bgl*II and *Hin*dIII, respectively. This plasmid DNA was then digested with *Hin*dIII and *Bgl*II to remove the simian virus 40 enhancer and promoter sequence. The plasmid pCATHB3 was constructed by ligation of the *Hin*dIII-*Bgl*II fragment excised from pHBV-3 into *Hin*dIII-*Bgl*II-digested pCAT DNA. Other deletion plasmids were prepared by *Bal* 31 treatment of *Hin*dIII-digested pCATHB3 or replacement of the *Hin*dIII-*Bgl*II fragment from pHBV-3 with that excised from a deletion mutant previously constructed for producing core particles.

CAT assay. Cell extracts were prepared by the method of Gorman et al. (10). CAT assays were carried out in each case with ^{14}C-labeled acetyl coenzyme A (Dupont, NEN Research Products, Boston, Mass.) used as the substrate. The efficiency of the transfer of the ^{14}C-labeled acetyl residue to chloramphenicol was determined by liquid scintillation counting. This assay system was suitable for quantifying relative CAT activities.

Assay of HBV core particle production. The transient expression system for HBV production has already been described (28). Deletion mutants of template HBV DNA were transfected to HuH-7 cells, and HBV core particles were prepared from the cytoplasms of the transfected cells. Crude core particle fractions were treated with sodium dodecyl sulfate (1%) and proteinase K (1 mg/ml); this was followed by electrophoresis on 1% agarose gels. The gel was subsequently blotted onto a nitrocellulose filter and hybridized with a ^{32}P-labeled HBV probe.

Blotting of mRNAs. Total mRNAs were prepared from HBV DNA-transfected cells by the guanidinium-cesium chloride method (5). Poly(A)$^+$ RNA fractions were obtained from an oligo(dT)-cellulose column. One microgram of each mRNA sample was electrophoresed on a 1% agarose gel containing 2.2 M formamide and transferred to nitrocellulose filter paper.

Nuclear extracts and electrophoretic mobility-shift assay. Extracts of HuH-7 cells were prepared by the procedure of Dignam et al. (6). Binding reactions were carried out in a final volume of 10 μl containing the following: 1 ng of a 5′-end-labeled *Hin*cII-*Rsa*I fragment (2.5×10^4 cpm), 10 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 100 mM KCl, and 5 μg of poly(dI-dC). Each reaction was initiated by the addition of 2 μl (approximately 5 μg of protein) of nuclear extract. Following 15 to 20 min of incubation at 37°C, the reaction mixture was subjected to 4% polyacrylamide gel electrophoresis in 6.6 mM Tris hydrochloride (pH 7.6)–1 mM EDTA–3.3 mM sodium acetate. All gels were dried and autoradiographed.

DNaseI footprinting. Binding reactions were scaled up threefold, and the amount of nuclear extract in each case was increased to 9 μl (22.5 μg of protein). After incubation for 15 min at 37°C, an equal volume of a mixture containing 5 mM $NaCl_2$ and 10 mM $MgCl_2$ was added, followed by digestion for 2 min at room temperature with 1 μl of freshly diluted solution (50 μg/ml) of DNaseI (DPFF; Worthington Diagnostics, Freehold, N.J.). Digestion was terminated by adding 2.5 μl of 250 mM EDTA, and the samples were

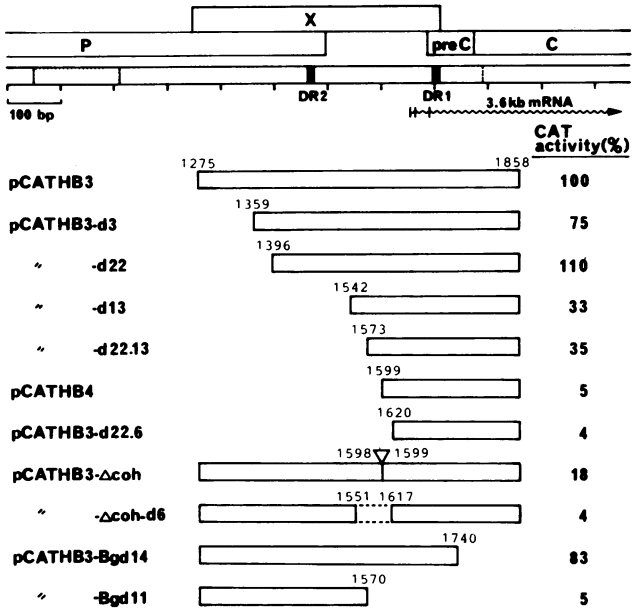

FIG. 1. Schematic representation of HBV DNA fragments inserted into CAT plasmids and CAT activities. Open boxes represent HBV DNA fragments ligated to a CAT gene. The numbers at the ends of the boxes indicate the nucleotide number from the sequence of HBV DNA (13). The open triangle indicates the insertion of an 8-bp *Sac*I linker DNA, and the dotted lines indicate the region of an internal deletion. The gene organization of the upstream region of the 3.6-kb mRNA is shown at the top of the figure. The relative CAT activity of each plasmid is indicated on the right.

electrophoresed as described above. Radioactive bands were identified by autoradiography, and the DNA fragments were eluted from the gel. The eluted DNAs were denatured and separated on a 7 M urea sequencing gel.

Methylation interference test. End-labeled fragments for the probe were treated with dimethyl sulfoxide, as described by Maxam and Gilbert (15). The methylated DNA was incubated in a standard binding reaction, and protein-complexed and unbound DNAs were separated on a 4% polyacrylamide gel. The DNA fragments were eluted from the gel and treated with 1 M piperidine for the cleavage of methylated G residues by the method of Maxam and Gilbert (15).

RESULTS

Identification of a promoter region for 3.6-kb mRNA transcription. Identification was made of the promoter sequence in the 5′-upstream region of the 3.6-kb mRNA transcriptional unit. A series of upstream regions of HBV DNA shortened by progressive deletions or mutated by a small insertion or internal deletion was ligated to the CAT gene, and the regions were compared for their expression in HuH-7 cells (Fig. 1). The relative CAT activity of each construction is indicated on the right of Fig. 1. Removal of 120 base pairs (bp) from nt 1275 to 1396 (pCATHB3-*d*22) failed to have any effect on expression. Deletion from nt 1396 to 1573 (pCATHB3-*d*22.13) caused a 70% drop in CAT activity. All residual activity was completely lost when deletion was carried out up to as far as nt 1599 (pCATHB4). It is thus evident that deletion decreases CAT activity in a stepwise manner. Insertion of an 8-bp linker DNA between nt 1598 and 1599 (pCATHB3-Δ*coh*) caused an 80% loss of CAT activity, and an internal deletion of 65 bp from nt 1552

to 1616 (pCATHB3-Δ*coh-d*6) resulted in the complete loss of activity. A deletion of 118 bp from nt 1740 to 1858 (pCATHB3-Bgd14) did not affect activity, but a larger deletion from nt 1570 to nt 1858 (pCATHB3-Bgd11) led to its complete loss.

From the data presented above, it is evident that the upstream region from nt 1573 to the start site of transcription of the 3.6-kb mRNA (nt 1657) is essential for the expression of promoter activity, and the sequence around nt 1598 is requisite for promoter function. The sequence from nt 1275 to 1570 was observed to have no promoter activity, while deletion of this sequence decreased CAT activity to some extent. This region thus appears to be involved in modulating transcription efficiency.

Promoter region essential for virus production. To confirm the significance of the identified promoter region in virus production, template plasmids for HBV production were constructed. All of these plasmids had similar deletions in the upstream region of the 3.6-kb mRNA start site, as described above. These templates for virus production were examined for their activity by assaying core particle production in the transient expression system by using HuH-7 cells. The template plasmid pHBV-3 was used as a wild type that had the sequence from the *Bam*HI site (nt 1275) in the upstream region (Fig. 2A and B). Core particles were noted to be produced by hybridization of replicative intermediate DNAs packaged within them (28). When pHBV-3*d*26 and -*d*13 mutants with deletions from nt 1275 to nt 1547 and to nt 1542, respectively, were examined, core particle production was detected, although to a lesser degree than that in pHBV-3. In contrast, no production could be found in either of the pHBV-4 or pHBV-3*d*27 mutants, in which deletion was carried out up to as far as nt 1599 and nt 1639, respectively. Furthermore, remarkable reduction was observed with the mutant pHBV-3Δ*coh*, which had a small insertion of 8 bp between nt 1598 and 1599 in pHBV-3. No production of core particles was detected in the internal deletion mutant pHBV-3Δ*coh-d*6, which had a 65-bp deletion from nt 1552 to 1616 in pHBV-3. It is thus evident that the essential sequence for virus production is located around nt 1598, and the upstream region between nt 1396 and 1542 is perhaps related to the efficiency of virus production.

The results of the virus production assay were in very close agreement with those of the CAT assay of the promoter function. It would thus appear that the identified promoter sequence is directly involved in replication of HBV through transcription of the 3.6-kb mRNA. In fact, as shown in Fig. 2C, 3.6-kb mRNA transcription was influenced by the Δ*coh* and Δ*coh-d*6 mutations. Northern hybridization of poly(A)$^+$ RNAs of transfected cells with the mutant templates showed the intensity of the 3.6-kb mRNA to decrease in the case of pHBV-2Δ*coh* (Fig. 2C, lane b), becoming less than that of pHBV-2 (Fig. 2C, lane a). The extent of 2.2-kb mRNA transcription remained the same, however. With pHBV-2 Δ*coh-d*6 (Fig. 2C, lane c), the 3.6-kb transcript virtually disappeared. As also shown in Fig. 2C, larger and smaller transcripts of about 4.1 and 3.1 kb appeared at the time of decrease in the 3.6-kb mRNA. Initiation sites of 4.1- and 3.1-kb RNAs were mapped to the upstream region of the X gene and at around the start position of the P gene, respectively (unpublished data), so that the initiation site of RNA transcription may change on introduction of the mutation into the original promoter region. Nevertheless, both 4.1- and 3.1-kb transcripts were not functional for virus production.

Unique structure of the promoter region. To clarify the

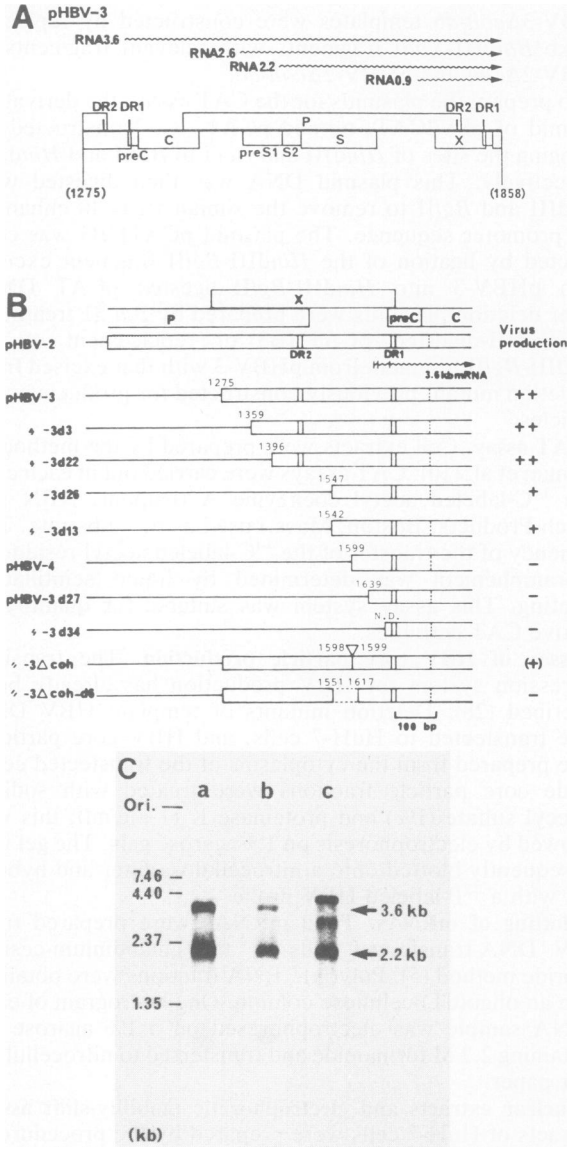

FIG. 2. (A and B) Structures of deletion mutants in the 5′-upstream region of the template HBV DNA and their activities for virus production. (A) The whole structure of the template plasmid pHBV-3. Its construction is described in the text. (B) Structures of deletion mutants. The open boxes and solid lines represent HBV DNA and pBR322 DNA, respectively. The numbers at the ends of the boxes indicate the nucleotide number of HBV DNA. N.D., Not determined. The linker insertion and internal deletion are the same as those described in the legend to Fig. 1. The results of the virus production assay are shown on the right of panel B. Symbols represent relative intensities of the hybridized HBV DNA: ++, efficient production; +, significant amount of virus production; (+), very reduced but still detectable level of virus production; −, not detectable. (C) Transcription of mutated template plasmids. The template plasmids were transfected into HuH-7 cells, and poly(A)$^+$ RNA fractions were prepared after 60 h. One microgram of each poly(A)$^+$ RNA was subjected to RNA blotting. A ^{32}P-labeled HBV DNA was used as the probe. Lane a, pHBV-2; lane b, pHBV-2Δ*coh*; lane c: pHBV-2Δ*coh-d*6.

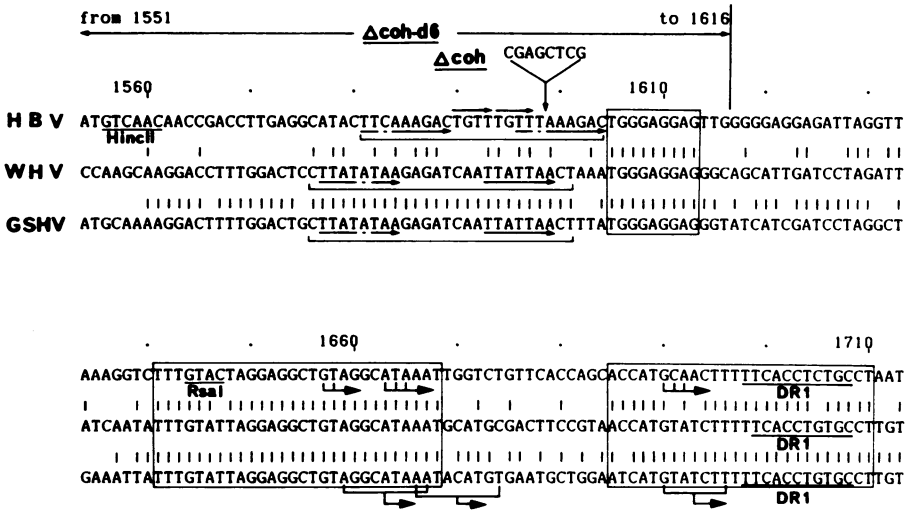

FIG. 3. Sequences of the 3.6-kb mRNA promoter region. The nucleotide sequence of the 3.6-kb mRNA promoter region of HBV DNA is shown along with the corresponding sequences of GSHV and WHV hepadnaviruses. The three sequences are arranged in the same order as that of the DR1 sequence. Vertical lines represent nucleotides common to HBV and WHV and to WHV and GSHV. The insertional mutations of Δ*coh* and Δ*coh-d6* are mapped on the HBV sequence. The small arrows indicate repeating sequences in AT-rich sequences. Similar repeating structures are shown in the sequences of WHB and GSHV. The boxed blocks indicate conserved sequences in the three hepadnavirus species. The large arrows correspond to the starting sites of the three species of the 3.6-kb mRNA.

structural features of the DNA sequence in the identified promoter region, the corresponding sequences of other hepadnaviruses, WHV (9) and GSHV (20), were compared with that of HBV (13) (Fig. 3). On arranging the sequences in the same order starting from DR1, an AT-rich sequence (from nt 1581 to 1604) containing a directly repeated sequence (TGTT) connected to the same flanking sequence PyAAAGAC (where Py is a pyrimidine) at both sides was found to be present. A corresponding region was conserved between WHV and GSHV, but not between HBV and WHV. The Δ*coh* mutation was located within this sequence and remarkably lessened promoter activity, indicating that this unique sequence is important for regulating 3.6-kb mRNA transcription in a species-specific manner. Three different completely conserved sequences among three species of hepadnaviruses were also found to be located between the AT-rich sequence and DR1, one being from nt 1605 to 1613, another being from nt 1641 to 1668, and the last being from nt 1685 to 1710 and including the DR1 sequence. Since three different start sites of transcription of 3.6-kb mRNA were similarly located in both HBV and GSHV, three conserved sequences may determine the initiation site of transcription in some way.

Identification of the specific binding factor of the essential promoter element. The AT-rich sequence in the promoter region identified as an essential element of the promoter function may be recognized by certain cellular factors. To examine this possibility, an electrophoretic mobility-shift assay was conducted on a nuclear extract of HuH-7 cells. Two DNA fragments were prepared as probes: an 87-bp *Hin*cII-*Rsa*I fragment from the wild-type HBV DNA and a similar 95-bp *Hin*cII-*Rsa*I fragment from the Δ*coh* mutant with an 8-bp linker insertion at the *Dra*I site within the AT-rich sequence. Figure 4A shows that a discrete band of a DNA-protein complex appears under conditions with and without 100 mM KCl when the wild-type probe is used. Although a discrete band was hardly observed by a short exposure (Fig. 4A), the complex formation became detectable by a longer exposure (data not shown), when the Δ*coh* mutant probe was used. It is thus clear that a factor binding to the AT-rich sequence is present and has been tentatively designated as 3.6-kb mRNA or pregenome promoter-binding protein.

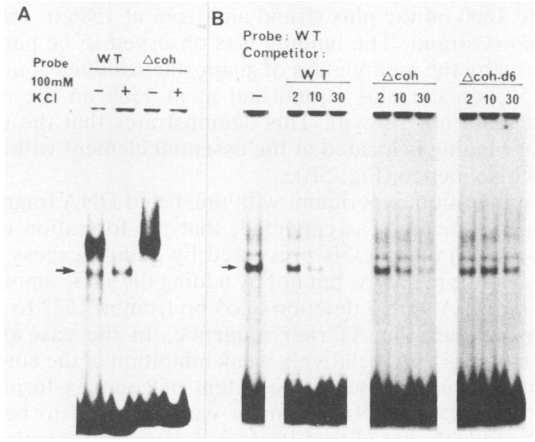

FIG. 4. (A) Detection of a specific factor binding to the promoter region by the electrophoretic mobility-shift assay. Two ^{32}P-labeled DNA fragments were used as probes. The wild-type (WT) probe was an 87-bp *Hin*cII-*Rsa*I fragment prepared from the wild type of HBV DNA, and the Δ*coh* probe was a 95-bp *Hin*cII-*Rsa*I fragment form the mutant plasmid pHBV-3Δ*coh* (Fig. 3). Binding reactions were carried out in both the presence and the absence of 100 mM KCl. The arrow indicates the position of the DNA-protein complex specific to the sequence of the wild-type HBV DNA. (B) Competition assay with mutant DNA fragments in the promoter region. The ^{32}P-labeled *Hin*cII-*Rsa*I fragment from wild-type HBV DNA was used as the probe. The *Bam*HI-*Bgl*II fragments of HBV DNA covering the promoter region were prepared from the wild-type plasmid or mutant plasmids and were used as competitors. The number above each lane is the molar ratio of competitor DNA to the probe. The assay was carried out in the presence of 100 mM KCl in the reaction mixture. The band from the specific interaction with the probe is indicated by the arrow.

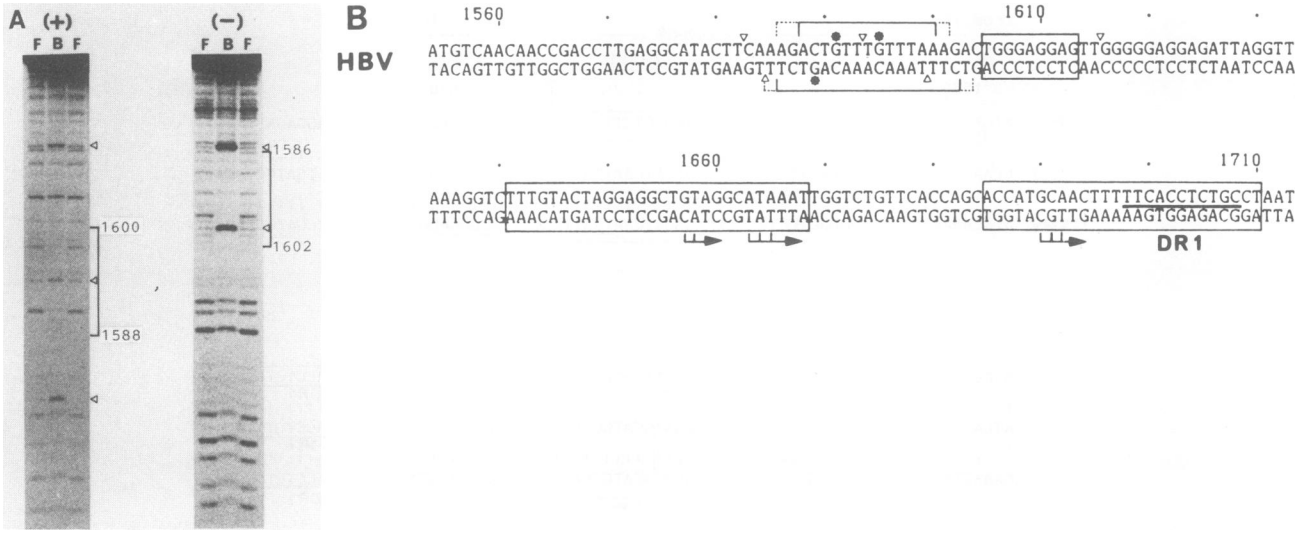

FIG. 5. Analysis of the target site of promoter-binding protein. The 5′-end-labeled 87-bp *Hin*cII-*Rsa*I fragment was used as the probe, and both the plus and minus strands were analyzed. (A) DNAase I footprinting. Protein-complexed DNA (B) and unbound DNA (F) were eluted separately and analyzed on an 8% polyacrylamide gel containing 7 M urea. The open triangles indicate DNase-hypersensitive bands induced by factor binding. (B) DNA sequence of the 3.6-kb mRNA or pregenome promoter-binding site. The binding sites derived from the DNase I footprinting analysis are indicated by the brackets on both strands. Asterisks indicate the positions of G residues where methylation interfered with the binding of the factor.

The sequence-specific binding of this factor was examined by DNase footprinting analysis and methylation interference experiments by using the 87-bp *Hin*cII-*Rsa*I DNA fragment as a probe and analyzing both the plus and minus strands. Figure 5A shows that the protection was provided from nt 1588 to 1600 on the plus strand and from nt 1586 to 1602 on the minus strand. The binding was observed to be partially hindered by the methylation of guanosine residues at nt 1591 and 1595 on the plus strand and at nt 1589 on the minus strand (data not shown). This demonstrates that the target site for binding is located at the essential element within the AT-rich sequence (Fig. 5B).

A competition experiment with unlabeled DNA fragments (Fig. 4B) indicated, as expected, that the formation of the DNA-protein complex is prevented by adding excess unlabeled, wild-type DNA, but not by adding the same amount of Δ*coh-d*6 DNA with a deletion of 65 bp from nt 1552 to 1616, which includes the AT-rich sequence. In the case of the Δ*coh* mutant DNA, relatively weak inhibition of the complex formation was observed. The extent of complex formation with the mutated DNA fragment was observed to be well correlated with that of the expression of promoter activity in the CAT assay, so that the binding of this factor to the essential element may be important for the promoter function.

To determine whether the factor binding to the 3.6-kb mRNA promoter was actually present in other cells, nuclear extracts from several other cell lines were prepared and assayed for factor binding by an electrophoretic mobility-shift assay. Figure 6 shows that a similar binding factor was present in HepG2 and huH2-2 cell extracts, while it was very scarce, if present at all, in those of HeLa and NIH 3T3 cells. The binding site of the factor from HepG2 cells was also determined and was found to be the same as that of HuH-7 cells (data not shown). That HuH-7 (17), HepG2 (12), and huH2-2 (27) cell lines were derived from human hepatomas indicates that this factor is possibly involved in the liver-specific regulation of transcription.

DISCUSSION

The 3.6-kb mRNA of HBV was previously reported to contain three species each with different 5′ ends (28), the shortest one probably being packaged in core particles and functioning as pregenome RNA for reverse transcription (8, 21). In the present study, the promoter region for the transcription of this 3.6-kb mRNA was identified. As also indicated by previous observations, the present results showed that the activity of the 3.6-kb mRNA promoter is very closely correlated with the extent of virus production.

In the promoter region of HBV, a unique AT-rich sequence was indicated by a comparison of the sequences of three hepadnaviruses: HBV, WHV, and GSHV. This sequence was confirmed by a CAT assay to be an essential element for functioning of the promoter. Since no well-

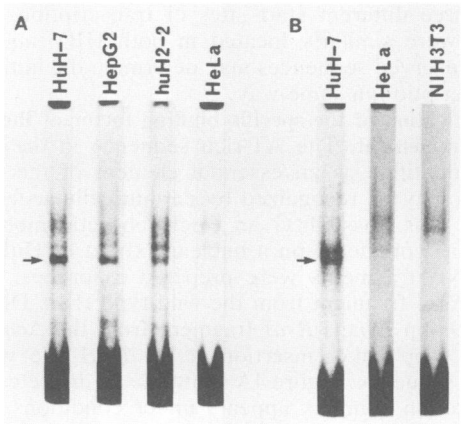

FIG. 6. Distribution of the promoter-binding protein. Nuclear extracts from the cells shown were prepared as described in the text. Binding reactions were carried out in 100 mM KCl. A and B indicate the results of independent experiments.

known promoter sequence, such as TATA and CAAT, could be found in this promoter region, the unique feature of the AT-rich sequence may be characteristic of the transcription of heterogeneous 3.6-kb mRNAs. There is no reliable means available for distinguishing 3.6-kb mRNAs from each other, and so it remains to be investigated whether there is specific regulation of the transcription of each mRNA.

The promoter region identified in this study does not include the so-called core promoter, as suggested by Rall et al. (19). These investigators located the initiation site of the core mRNA of HBV DNA at nt 1640 ± 50 in their sequence (nt 1510 ± 50 in our sequence) by an in vitro transcription system using HeLa cell extracts. However, we could find no such transcript in either liver cells infected with HBV in vivo (26) or the transient expression system for HBV production (28). In addition, it has been observed that HeLa cells are unsuitable for transcription of the HBV genome because of lack of the ability to efficiently carry out the transcription of HBV in the transient expression system (unpublished data). Thus, the core promoter suggested by Rall et al. (19) is not essential to 3.6-kb mRNA transcription in vivo.

In the present study a nuclear factor interacting with the essential element for functioning of the promoter was also identified. The binding of this factor was observed to be hindered by the Δ*coh* mutation within the AT-rich sequence, and this was closely correlated with a reduction in promoter activity by the same mutation. Binding of the factor to the essential element thus appears to be possibly involved directly with the promoter function. A similar binding factor was found in nuclear extracts from HepG2 and huH2-2 hepatoma cells, but not from HeLa or NIH 3T3 cells. The efficient transcription and replication of HBV were observed only in HuH-7 and HepG2 cells, both of which are known to be highly differentiated hepatoma cell lines. This binding protein can thus reasonably be considered to be a liver-specific factor which stimulates HBV transcription.

The binding factor was also shown to be present in the extract of huH2-2 cells, although transient transcription of HBV DNA was not observed in huH2-2 cells by DNA transfection (28). huH2-2 cells contain a single copy of subgenomic HBV DNA integrated into the chromosome and are incapable of transcribing integrated HBV DNA (27). However, this integrated form becomes transcriptionally active on being introduced into HuH-7 cells by DNA transfection (unpublished data), and thus, certain factors that dominantly block the transcription of HBV appear to be present in huH2-2 cells, or huH2-2 cells may lack an essential factor among the multiple components necessary for HBV transcription.

Another factor that binds to the promoter region was also indicated by the results in Fig. 4A and could be observed only at low ionic strength. Its target sequence was located from nt 1626 to 1641, between two conserved sequence blocks. This factor was found to be present even in HeLa and NIH 3T3 cell extracts (unpublished data), and thus can reasonably be concluded to be a ubiquitous factor that is involved in the promoter function and binding of the specific factor. The exact role of this factor remains to be elucidated.

Transcription and replication of HBV were observed to occur most efficiently in liver cells, indicating that liver cell-specific regulation is possibly responsible for the expression of HBV DNA. Since the specific binding protein identified in this study was of cellular origin, it follows that this protein (the 3.6-kb mRNA or pregenome promoter-binding protein) should be involved in the transcription of cellular genes in liver cells. The purification and characterization of this protein are now being conducted and may provide some clarification as to how interactions between HBV and cellular genes in hepatocytes and gene expression of HBV DNA are regulated.

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