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A Transcription Initiation Site for the Hepatitis B Virus X Gene Is Directed by the Promoter-Binding Protein

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Recent studies have demonstrated the transacting function of the X gene product of hepatitis B virus. However, little information is available on the regulation of X gene expression. In this report, we first investigate a cellular factor regulating X gene transcription by DNA transfection, using the human hepatoma cell line HuH-7, which is permissive for HBV replication as well as X mRNA transcription. A sequence-specific cellular factor was found to bind to the promoter region upstream of the first ATG (nucleotide [nt] 1248) of the X open reading frame. DNase I footprinting analysis showed the binding sequence of this factor to be situated between nt 1097 and 1119, where an 8-bp palindrome structure resides. S1 nuclease analysis of X gene transcripts demonstrated the binding site to be adjacent to two major start sites (nt 1117 and 1125) of X mRNA. Second, we demonstrate that introduction of a mutation into the binding site gives rise to a loss of the binding with a concomitant shift of the transcription start site of X mRNA beyond the 8-bp palindrome structure, causing it to become more heterogeneous. Thus, the promoter-binding protein appears to be involved in directing the transcription initiation site of the X gene toward the downstream region of the X promoter when X protein is produced from X mRNA.

The genome of hepatitis B virus (HBV) contains four overlapping open reading frames (ORFs) (29). Three of these have been assigned for the determination of viral proteins: viral surface protein (preS-S), viral core protein (C), and viral polymerase (P). The fourth ORF, X, is believed to code for a protein that alters the DNA-binding specificity of CREB and ATF2 (15) and possesses homology with a unique domain structure of known serine protease inhibitors (27, 28). Cotransfection experiments with X expression plasmids and reporter plasmids bearing chloramphenicol acetyltransferase (CAT) genes under the control of various promoters indicated a transactivating effect of the X gene product (14, 22, 26, 31). Two different features of the X gene product may appear to contribute together to liver carcinogenesis by activation of cellular genes in *trans*. However, only a small amount of X mRNA has been detected in liver tissues chronically infected with HBV (3). This low expression of the X gene is in marked contrast to observations on the transient expression of the HBV genome in a cultured-cell system (35). Thus, so far, only a little information is available on the regulation of X gene expression.

In this study, we examined the regulation of X gene transcription in the virus life cycle by using the permissive cell line HuH-7 for virus production by DNA transfection (35). A cellular factor regulating the initiation of X gene transcription was sought, and the specific binding protein interacting with the promoter region adjacent to the 5′ end of the X gene mRNA was identified. Mutations in the binding sequence resulted not only in reduction of the binding activity of this factor with a concomitant loss of the X gene transcription (18) but also in a shift of the transcription start site of X mRNA to the upstream region of the binding site. The promoter-binding protein thus appears essential to direct a start site of X gene transcription downstream of the X promoter to bring about the translation from the 0.9-kb X mRNA but from no other viral transcripts (see Fig. 1A) in the viral life cycle when X protein is produced. On the other hand, the transcription start sites of the X promoter-CAT construct are located upstream of the 8-bp palindrome sequence, although the promoter-binding protein is bound to it (18).

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MATERIALS AND METHODS

Construction of expression plasmid DNAs. Construction of plasmid pHBV-3 has been described previously (34). Plasmid pHBV-3 contains 1.2 copies of the whole HBV DNA (Fig. 1A), which direct the production of virus particles in the cell culture system as shown by DNA transfection experiments. Plasmid pHBVX-1, as also reported previously (35), contains the 0.86-kb *Stu*I-*Bgl*II fragment (nucleotides [nt] 987 to 1858) (13) covering the X ORF and upstream regulatory sequences (see Fig. 1A).

Plasmids pHBE-17, pHBE-18, and pHBE-22 are subclones containing *Bal*I-*Mbo*I-digested fragments E-17 (nt 1136 to 1274), E-18 (nt 1091 to 1136), and E-22 (nt 989 to 1136), respectively, of the 0.29-kb *Bam*HI-*Stu*I fragment (nt 1274 to 989). Each *Bal*I-*Mbo*I fragment was inserted into the *Hin*dIII site of pBR322 by previously ligating the *Hin*dIII linker DNA.

Mutant plasmids pHBVX-sph6 and pHBVX-sph16 were constructed from pHBVX-1 by inserting the 8-bp *Sac*I linker DNA and the 8-bp *Bgl*II linker DNA, respectively; into the *Sph*I site (nt 1110). The 3′ overhanging of the *Sph*I site was treated with T4 DNA polymerase. Plasmid pHBV-3-sph6 was made from pHBV-3 by insertion of the 8-bp *Sac*I linker DNA into the *Sph*I site.

Cell lines. HuH-7 (17, 35), huH2-2 (33), and HLEC-1 (6) were derived from human hepatocellular carcinomas. HepG2 (12) was derived from human hepatoblastoma. HuH-7, HepG2, and HLEC-1 cells were negative for HBV integration, whereas huH2-2 cells had a single integration of

HBV subgenomic DNA (33). HeLa and NIH 3T3 cells were used as nonliver cells.

Preparation and blotting of RNA. Total RNAs were prepared from DNA-transfected cells by the guanidinium-cesium chloride method (4). Poly(A)$^+$ fractions were obtained by oligo(dT)-cellulose column chromatography. RNA samples were electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose filter.

Preparation of nuclear extracts and electrophoretic mobility shift assay. A nuclear extract of each cell line was prepared by the procedure of Dignam et al. (5). Binding reactions were carried out in a final volume of 10 μl containing 1 to 2 ng of a 5′-end-labeled *Bam*HI-*Stu*I fragment (2×10^4 cpm), 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 5 μg of sonicated salmon sperm DNA. Each reaction was initiated by the addition of 2 to 4 μl (5 to 10 μg of protein) of nuclear extract. The reaction mixture was incubated for 15 to 20 min at 37°C and subjected to polyacrylamide gel electrophoresis (4% acrylamide) in 6.6 mM Tris-HCl (pH 7.6)–1 mM EDTA–3.3 mM sodium acetate. After electrophoresis, the gels were dried and autoradiographed.

DNase I footprinting. As previously described (34), binding reactions were scaled up threefold and the amount of nuclear extract was increased to 9 μl (ca. 22 μg of protein). The ^{32}P-labeled *Stu*I-*Bam*HI fragment of HBV DNA and nuclear extract were incubated for 15 to 20 min at 37°C. After incubation, an equal volume of a mixture containing 5 mM NaCl and 10 mM $MgCl_2$ was added, followed by digestion for 2 min at room temperature with 1 μl of a freshly diluted solution (50 μg/ml) of DNase I (DPFF; Worthington Diagnostics, Freehold, N.J.). Digestion was terminated by adding 2.5 μl of 250 mM EDTA, and the samples were 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 (16).

S1 nuclease analysis. Appropriate restriction fragments corresponding to the upstream region of the X ORF were prepared from pHBVX-1 DNA and mutant plasmids derived from pHBVX-1. Each fragment was labeled at the 5′ end with T4 polynucleotide kinase and digested with a secondary restriction enzyme to obtain 5′-end-labeled probe DNA. S1 nuclease analysis was carried out as described previously (8). The protected products were subjected to polyacrylamide gel electrophoresis in a gel containing 7 M urea. The product size was estimated by comparison with size markers consisting of labeled *Hin*fI-digested fragments of pBR322.

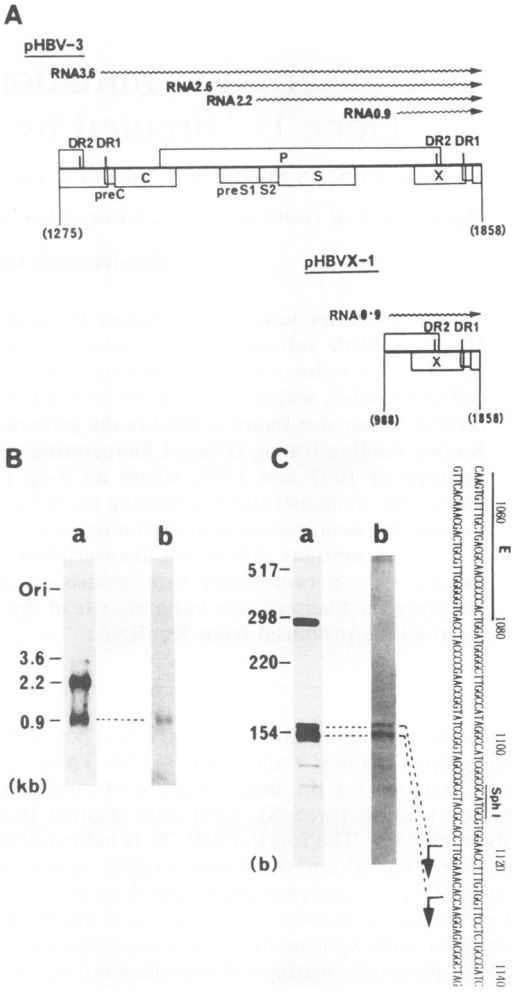

FIG. 1. Analyses of X gene transcripts. (A) Structures of template plasmids for X gene transcription. (B) RNA blotting of X gene transcripts. A 1-μg sample of poly(A)$^+$ RNA from each transfectant was subjected to RNA blotting. The ^{32}P-labeled whole genome of HBV DNA was used as a probe. Lanes: a, pHBV-3; b, pHBVX-1. (C) S1 nuclease analysis of X gene transcripts. The ^{32}P-end-labeled 0.29-kb *Bam*HI-*Stu*I HBV DNA prepared from pHBV-3 (lane a) and the ^{32}P-end-labeled 0.64-kb *Bam*HI-*Hin*dIII fragment prepared from pHBVX-1 DNA (lane b) were used as the probes. The S1 product size was estimated, and the 5′ ends of the transcripts were mapped on the sequence of HBV DNA (13). Arrows indicate major transcription start sites of the X gene transcript.

RESULTS

X gene transcripts. To analyze the characteristics of the X gene transcript, the X gene was expressed in HuH-7 cells, which are permissive for the production of HBV particles (35), by DNA transfection experiments with two types of template plasmids, pHBV-3 and pHBVX-1 (Fig. 1A). Plasmid pHBV-3 contains 1.2 copies of the whole genome of HBV, which direct the production of virus particles in the cell culture system. pHBVX-1, containing a 0.86-kb *Stu*I-*Bgl*II fragment of HBV DNA, was constructed only for expression of the X gene. These plasmids were transiently expressed, and the resulting 0.9-kb poly(A)$^+$ RNA from each transfectant was subjected to RNA blotting analysis (Fig. 1B). The 0.9-kb RNA transcript (RNA0.9 in Fig. 1A) derived from the X gene was observed in both cases. Transcription of the X gene was much more efficient for pHBV-3 than for pHBVX-1. S1 nuclease mapping of the 5′ end of the 0.9-kb X mRNA (Fig. 1C) showed two major start sites and other minor start sites heterogeneously located downstream of the two major start sites. The two major start sites of X mRNA were mapped at nt 1117 ± 3 and 1125 ± 3 on the HBV DNA sequence of subtype adr, where the first ATG of the X ORF is located at nt 1248 (13). From S1 mapping data, no difference in transcription start sites could be observed between two templates, pHBV-3 and pHBVX-1. These major start sites were located about 60 bp upstream from those previously reported by Treinin and Laub (30).

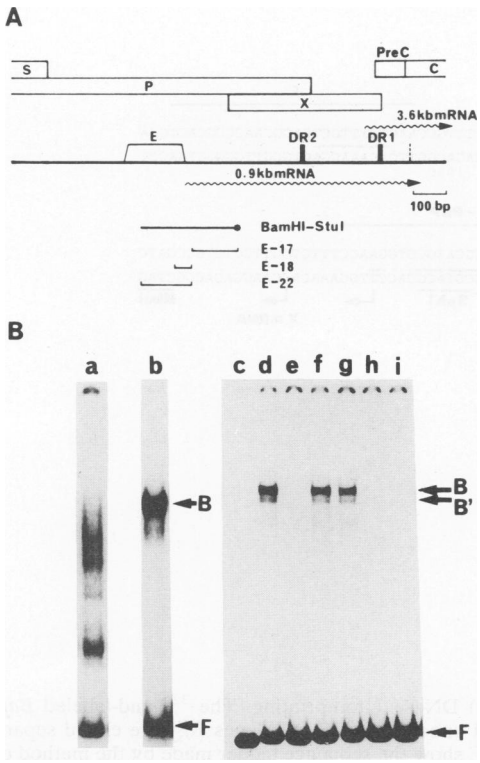

FIG. 2. Identification of sequence-specific binding protein to the promoter region of the X gene. (A) Schematic representation of the genetic structure of the X gene and its upstream and downstream regions. E represents the enhancer region. The ^{32}P-end-labeled *Bam*HI-*Stu*I fragment (nt 1274 to 989) was used as the probe for the electrophoretic mobility shift assay. Subfragments E-17, E-18, and E-22 were cloned into pBR322 and used for the competition assay. (B) Results from the electrophoretic mobility shift assay. As nonspecific competitor DNAs, 3 μg of poly(dI-dC) DNA (lane a) and 5 μg of sonicated salmon sperm DNA (lane b) were examined. The competition assay was carried out by adding 1 μg each of various plasmids to the reaction mixture as follows: lane c, no nuclear extract; lane d, no competitor DNA; lane e, pHBVX-1; lane f, pBR322; lane g, pHBE-17; lane h, pHBE-18; lane i, pHBE-22. The major and minor shifted bands were designated as B and B′, respectively. F indicates an unbound free DNA.

Sequence-specific binding protein to the promoter region of X gene. To find a specific cellular factor interacting with the upstream region of the X ORF, we performed an electrophoretic mobility shift assay with a 0.29-kb *Bam*HI-*Stu*I DNA fragment (nt 1274 to 989) as the probe (Fig. 2A). This fragment contains the upstream sequence of the X ORF and two-thirds of the enhancer region. First, two types of nonspecific competitor DNAs, a double-stranded poly(dI-dC) and a sonicated salmon sperm DNA, were examined for the assay. As shown in Fig. 2B, lanes a and b, the use of double-stranded poly(dI-dC) led to a complex pattern of many shifted bands, possibly as a result of cellular factors known to interact with the enhancer region (1, 7). In contrast, only one major shifted band was observed when sonicated salmon sperm DNA was used instead of double-stranded poly(dI-dC).

Plasmids containing subfragments (E-17, E-18, and E-22, as described in Fig. 2A) derived from the 0.29-kb *Bam*HI-*Stu*I HBV DNA fragment were prepared and used for the electrophoretic mobility shift assay as competitors (Fig. 2B). Competition was clearly evident following addition of pHBVX-1 (lane e), pHBE-18 (lane h), or pHBE-22 (lane i) but not pHBE-17 (lane g) or pBR322 (lane f). The binding of this factor is thus shown to be specific to the 47-bp *Bal*I-*Mbo*I DNA fragment (nt 1091 to 1136) upstream of the X ORF.

DNase I footprinting experiments were carried out to locate the binding site precisely on the HBV DNA sequence. The sequence between nt 1097 and 1119 was protected by the binding of this factor (Fig. 3A, lane B). This target sequence had an 8-bp inversely repeated structure containing the *Sph*I site at its center (Fig. 3B) and was located above the 47-bp sequence. In Fig. 2B, besides this major shifted band (B), a minor band (B′) also appears. Footprinting analysis of this band gave almost the same results as those for the major band, as shown in Fig. 3A, lane B′. A minor factor interacting with the same sequence appears to be present but differs in electrophoretic mobility. Mapping of the binding site indicated that the cellular factor interacts with the sequence immediately adjacent to the 5′ end of the X gene transcript (Fig. 3B). This factor may be considered to be involved in the initiation of X gene transcription. In separate experiments, a chemically synthesized DNA fragment containing the above 47-bp sequence had promoter activity, as shown by the CAT assay method (18). This factor was thus tentatively named X-promoter-binding protein (X-PBP in Fig. 3B) (18).

The distribution of X-PBP was examined by using the cell lines HuH-7, HepG2, huH2-2, HLEC-1, and HeLa. Nuclear extracts of these cells were prepared and tested by electrophoretic mobility shift assay (Fig. 4). In the absence of specific competitor pHBVX-1 DNA (− lanes), but not in the presence of a specific competitor (+ lanes), all the extracts showed similar shifted bands. Besides these human cell lines, the mouse fibroblast cell line NIH 3T3 was also observed to possess a similar factor (data not shown). Data indicate that this X-PBP is generally present in mammalian cells.

Insertion mutation in the binding sequence of X-PBP. To account for the physiological functions of X-PBP, we introduced insertion mutations into the binding sequence of X-PBP and examined their effects on X-PBP binding and transcription of the X gene. Two mutant plasmids, pHBVX-sph6 and pHBVX-sph16, were prepared by the insertion of an 8-bp *Sac*I linker DNA and an 8-bp *Bgl*II linker DNA, respectively, into the *Sph*I site of pHBVX-1. Interaction of X-PBP with these mutant DNAs was analyzed by electrophoretic mobility shift assay with DNA fragments prepared from mutant plasmids as probes. The results clearly showed no shifted band with the same amount of the mutant DNA fragments (Fig. 5A, lanes b and c). A competition experiment was also performed with each mutant plasmid as a competitor in the electrophoretic mobility shift assay. Two mutant plasmids were shown not to be competitive for binding (Fig. 5B, lanes c and d for pHBVX-sph16 and pHBVX-sph6, respectively). It was thus concluded that both insertion mutations at the *Sph*I site prevent the binding of X-PBP to the promoter region. That X-PBP binding was hardly detectable for pHBVX-sph16 (Fig. 5A, lane c), in which the insertion mutation was a 4-bp inversely repeating sequence (GATC) at the center of the palindrome structure, indicates that not only the sequence but also its configuration is important for the interactions of X-PBP.

Initiation site of X gene transcription directed by X-PBP interactions. To further elucidate roles of the palindrome

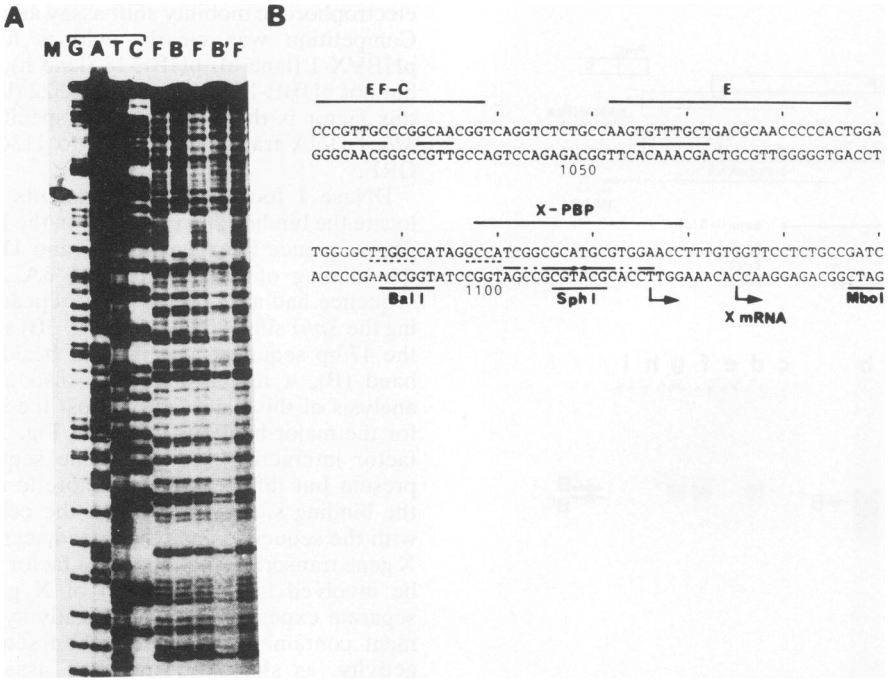

FIG. 3. Mapping of the target sequence of the specific binding protein. (A) DNase I footprinting. The ^{32}P-end-labeled *Bam*HI-*Stu*I fragment was used as the probe. Protein-complexed DNA (lanes B and B') and unbound free DNA (lanes F) were eluted separately and analyzed on an 8% polyacrylamide gel containing 7 M urea. The four lanes GATC show the sequence ladder made by the method of Maxam and Gilbert (16). Lane M shows a *Bal*I-digested probe DNA. (B) Map of the binding site. The binding site of X-PBP is indicated by the solid line over the DNA sequence. The inversely repeated structure is indicated by small arrows between complementary strands. The binding sequences of other protein factors are shown as EF-C and E. Dotted lines around the *Bal*I site indicate the consensus sequence for NF-I binding (1). This X-PBP binding is shown to be distinct from the NF-I binding. The two major start sites of X mRNA are shown by large arrows under the DNA sequence.

structure and X-PBP, we investigated the transcription start site of X mRNA with pHBVX-1, pHBV-3, and their mutant plasmids. Each template plasmid was transiently expressed by DNA transfection to HuH-7 cells, and each RNA transcript was subjected to S1 nuclease analysis. As shown in Fig. 6A, the S1 products from two mutants (pHBVX-sph16 and pHBVX-sph6) became larger and more heterogeneous than those of the wild-type pHBVX-1. On the basis of S1 product size, the start sites of RNA transcripts were mapped on the HBV DNA sequence (Fig. 7). The transcription start sites shifted to the upstream region of the X-PBP-binding site and became distributed in a heterogeneous manner. The distribution of shifted start sites was almost the same in both mutant DNAs, except for one strong band observed only for pHBVX-sph16 (Fig. 6A). Agreement of the results for the two mutants suggests that the shift of the transcription start sites may be due to the weak interactions of X-PBP. Interestingly, Shaul and Ben-Levy (23) found start sites to be mapped within the E element of the HBV enhancer under their conditions (Fig. 7). This implies that the E element may be engaged in the regulation of X gene transcription as well as enhancer activity.

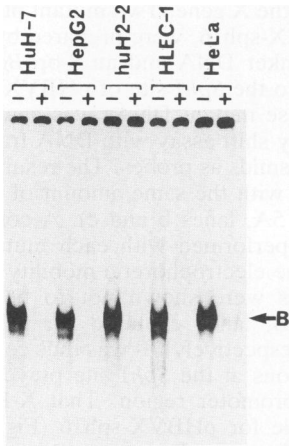

FIG. 4. Identification of the cellular factor X-PBP in the human cell lines. Nuclear extracts from several human cell lines were examined for similar binding activity. The ^{32}P-end-labeled *Bam*HI-*Stu*I fragment (nt 1274 to 989) was used as the probe. The minus and plus signs represent the absence and presence, respectively, of 1 μg of pHBVX-1 DNA as the specific competitor.

We prepared another mutant plasmid, pHBV-3-sph6, to determine whether mutation at the X-PBP-binding site really affects X gene transcription in the virus replication cycle. The mutant pHBV-3-sph6 was constructed by introducing the same mutation as that in pHBVX-sph6 into pHBV-3 DNA. The X gene transcript from the mutant template was examined by S1 nuclease analysis and compared with that of the wild-type template, pHBV-3 DNA. A shift of the transcription start sites was also observed in mutant template pHBV-3-sph6 (Fig. 6B). More shifted bands of S1 products were observed than for pHBVX-sph6 (Fig. 6A), even though the same mutation is present in both mutants with respect to the X-PBP-binding site. The reason for this is probably

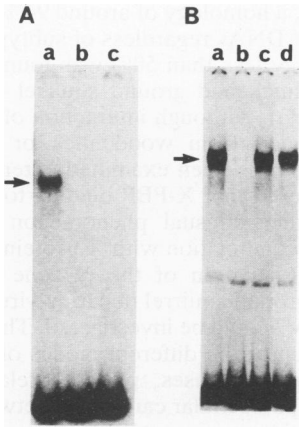

FIG. 5. Binding of X-PBP to the mutant DNAs. (A) The electrophoretic mobility shift assay was carried out with wild-type and mutant probes. The 0.33-kb ^{32}P-end-labeled *Sac*II-*Hin*cII fragment (nt 1325 to 1009) of HBV DNA (13) was prepared from each plasmid DNA and used as the probe. Lanes: a, pHBVX-1; b, pHBVX-sph6; c, pHBVX-sph16. (B) Competition assay for the X-PBP binding. The wild-type DNA fragment was used as the probe, and mutant DNAs were added as competitors. Lanes: a, no competitor DNA; b, pHBVX-1 (1 μg); c, pHBVX-sph16 (1 μg); d, pHBVX-sph6 (1 μg).

a difference between the template structures, the whole HBV genome in pHBV-3 and its subgenomic fragment in pHBVX-1. Nevertheless, the transcription start sites of X mRNA also shifted to the upstream region of the X-PBP-binding site in the virus replication cycle, when X-PBP binding to the X promoter region was reduced under certain conditions.

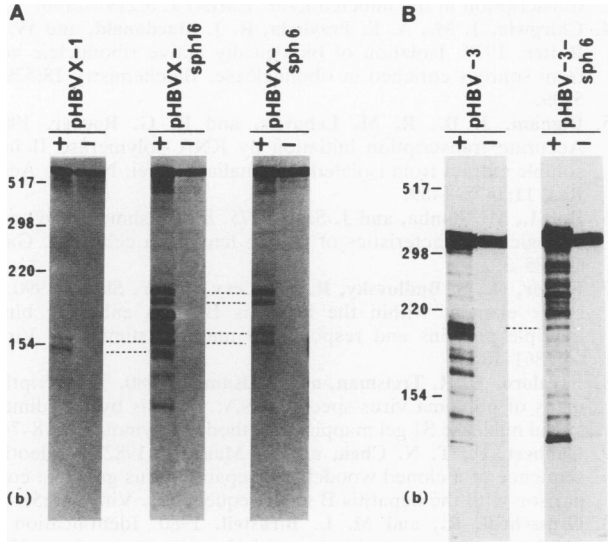

FIG. 6. S1 nuclease analyses of X gene transcripts of the mutant template plasmids. (A) Total RNA was prepared from each transfected cell line with wild-type template (pHBVX-1) or mutant templates (pHBVX-sph16 and pHBVX-sph6) and subjected to S1 nuclease analysis. The 0.64-kb *Bam*HI-*Hin*dIII fragment was prepared from each template plasmid. These fragments were labeled at the *Bam*HI site (nt 1274) and used as probes. (B) The X gene transcript in the virus replication cycle was analyzed by using the wild-type or mutant template (pHBV-3 and pHBV-3-sph6, respectively). The 0.33-kb *Sac*II-*Hin*cII HBV fragment (nt 1325 to 1001) was prepared from each template plasmid and used as the probe. The plus and minus signs represent the presence and absence, respectively, of RNA samples in the S1 reaction mixture.

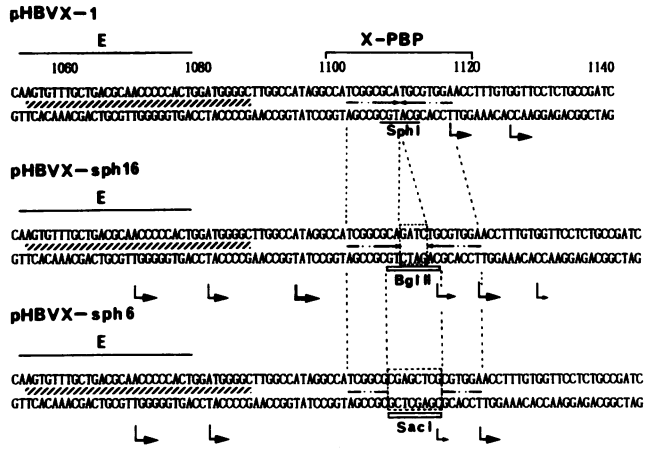

FIG. 7. Nucleotide sequences of the promoter region of X gene and the transcription start sites. Nucleotide sequences of the promoter region in pHBVX-1 and the mutant plasmids pHBVX-sph16 and pHBVX-sph6 are shown. Nucleotide numbers are according to the HBV DNA sequence of subtype adr (13). Inserted linker DNAs are indicated by open boxes under the sequences of mutant DNAs. Arrows indicate the positions of the 5′ ends of X gene transcripts, determined from the results of S1 nuclease analysis (Fig. 6A). The shaded area between complementary strands represents the completely conserved sequence stretch in the enhancer region in hepadnaviruses.

DISCUSSION

Possible role of X-PBP in X gene transcription. In this study we analyzed X gene transcription of HBV by using a human hepatoma cell line, HuH-7, as a recipient for DNA transfection. HuH-7 cells are permissive for productive HBV replication and X protein production (35). The results obtained are thus considered to reflect transcription of the X gene in liver tissue. The two major start sites of X gene transcripts are nt 1117 ± 3 and nt 1125 immediately adjacent to the downstream region of the X gene promoter (18). Transcription initiation from the upstream region, however, could hardly be detected (Fig. 1). These major start sites were at the downstream boundary of the enhancer region (1, 24), situated about 60 bp upstream of the sites reported previously by Treinin and Laub (30). They also indicated that the promoter region of the X gene transcription just overlaps the enhancer sequence.

We found a cellular factor which specifically interacts with the 23-bp sequence adjacent to the start sites of X mRNA (Fig. 3), and this target sequence was located in the 47-bp sequence (nt 1091 to 1136) possessing promoter activity (18). The factor was thus designated as X-PBP. It was shown to be present ubiquitously. Although the physiological functions of X-PBP were not fully investigated, the following two explanations are suggested. One is that X-PBP itself is a common cellular component of the transcription apparatus. However, no homologous sequence with the X-PBP-binding site has been found in other target sequence of cellular transcription factors so far identified. The second is that X-PBP is involved in transcription by interacting with some component of the transcription machinery including X pro-

tein. The mutations introduced into this target sequence for the X-PBP binding shifted the transcription start sites beyond the palindrome structure in the X-PBP-binding site and allowed them to be more heterogeneous. The binding of X-PBP is thus shown to be essential for directing the initiation sites of X gene transcription, which permit X protein synthesis from the X mRNA but not from other viral transcripts, as shown in Fig. 1A. In contrast, the similar mutations introduced into this target sequence for X-PBP binding failed to shift the transcription start site of the CAT construct, in which the X promoter region was directly connected upstream the CAT ORF (18) and the transcription start sites were found to be located upstream the palindrome sequence, indicating a direct role of X-PBP together with some cellular factor(s) in the regulation of X gene expression.

To determine whether two different insertion mutations introduced at the X-PBP-binding site alter the X gene transcription, the X gene transcripts from the mutant templates pHBVX-sph16 and pHBV-sph6 were also examined by S1 nuclease analysis with roughly the same amount of total RNA in each case and compared with that of the wild-type template, pHBVX-1. The intensity of the protected bands was not significantly reduced in the mutant templates compared with the wild-type template (unpublished data). This result is in contrast to the previous observation, in which transcription of the mutant X promoter-CAT construct (18) was reduced because of the mutation in the X-PBP binding site. The exact reason for this difference in the two systems is not known yet. However, X protein could be synthesized in the present system but not in the previous system. This difference may cause the conflicting yet interesting result. More detailed studies remain to be carried out.

X-PBP-binding site as a possible initiator sequence. In general, elements in the promoter region can be divided into two groups: initiators, which direct the initiation of transcription from a specific start site, and modulators, which influence the rate of transcription. Among the elements for transcription initiation, the TATA box is the most common sequence; it is located about 30 bp upstream from the transcription start site (2). Mutagenesis of the TATA sequence results in decreased initiation frequency (11, 32) and/or initiation from more heterogeneous sites (10, 11). The transcription factor TFIID has been identified as a specific binding factor which interacts with the TATA box (20). Other initiator sequences have recently been found in the murine terminal deoxynucleotide transferase gene, in the adenovirus major late promoter (25), and in the glial fibrillary acidic protein gene (19). These initiators are located at the transcription start site or downstream from the start site. Similar to the TATA box and factor TFIID, some protein factor may interact with an initiator sequence as a component of the transcription apparatus. Thus, although no homology has been found between the X-PBP-binding sequence and the known initiator sequence, the sequence stretch including the X-PBP-binding site may be another type of initiator sequence. Direct examination of the activity as an initiator has yet to be made.

Differences between X-PBP-binding sequences among hepadnaviruses. In contrast to the ubiquitous presence of the cellular factor X-PBP, the target sequence of X-PBP in HBV DNA was found to be unrelated to that of woodchuck (9) or ground squirrel (21) hepatitis virus. When the X-PBP-binding sequence of HBV DNA (subtype adr) was compared with the corresponding nucleotide sequences among other hepadnaviruses, a homology of around 94% was observed in the human HBV DNAs regardless of subtypes; on the other hand, a homology less than 50% was found between HBV and the woodchuck and ground squirrel hepatitis viruses (unpublished data). Although interaction of X-PBP with the X promoter region from woodchuck or ground squirrel hepadnavirus has not been examined extensively, it has so far been considered that X-PBP binding to the X promoter region is a rather unusual phenomenon in the X gene transcription in combination with X protein and X responsible elements. Regulation of the X gene transcription of woodchuck or ground squirrel hepatitis virus in comparison with that of HBV should be investigated. This would provide some clarification of the different modes of X gene expression among hepadnaviruses, possibly related to different incidences of hepatocellular carcinoma between humans and rodents.

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