JOURNAL OF VIROLOGY, Sept. 1989, p. 3919–3925
0022-538X/89/093919-07$02.00/0
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Vol. 63, No. 9

Characterization of Hepatitis B Virus Major Surface Antigen Gene Transcriptional Regulatory Elements in Differentiated Hepatoma Cell Lines†

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Received 19 April 1989/Accepted 8 June 1989

The regulatory DNA sequence elements that control the expression of the hepatitis B virus major surface antigen gene in the hepatoblastoma cell line HepG2 were analyzed by using transient transfection assays. In this system, the hepatitis B virus enhancer increases transcription from the surface antigen promoter approximately twofold. The promoter elements regulating the expression of this gene are within a 200-nucleotide sequence located immediately upstream of the transcription initiation sites. The promoter consists of an 85-nucleotide distal element which increases transcription from the surface antigen gene by two- to fourfold and a proximal element of approximately 115 nucleotides which is essential for transcriptional activity. The proximal and distal promoter elements were shown to bind factors present in HepG2 nuclear extracts, which is consistent with the regulatory role demonstrated for these sequences. The regulatory role of these promoter sequences in the hepatocellular carcinoma cell lines PLC/PRF/5 and Hep3B was also demonstrated, indicating similar transcriptional regulation of the surface antigen gene in each of these differentiated hepatoma cell lines.

The hepatitis B virus (HBV) genome is a circular 3.2-kilobase partially double-stranded DNA molecule (24). In the virion, the genome is enclosed in a 27-nm nucleocapsid of HBV core antigen which is enveloped by a lipoprotein coat comprising cellular lipid and HBV surface antigen (35). During HBV infection, 3.5-, 2.4-, 2.1-, and possibly 0.7-kilobase RNAs are transcribed from the viral genome (4, 5, 16, 18, 32, 42). These transcripts code for the nucleocapsid polypeptides and putative HBV polymerase, the large surface antigen polypeptide, the middle and major surface antigen polypeptides, and possibly the X-gene product, respectively (35). The regulatory sequence elements that control the expression of these transcripts have been characterized in a variety of cell lines. These analyses have indicated that the HBV genome contains a transcriptional enhancer element located between the 3′ end of the surface antigen gene and the 5′ end of the X-gene open reading frame (ORF) (1, 3, 7, 13, 14, 17, 29, 36, 40). In addition, regulatory sequence elements have been identified near the transcription initiation sites of each of the HBV RNAs (9, 15, 19, 23, 28, 30, 31, 37).

Recently, it has been demonstrated that certain hepatoma cell lines have the capacity to complete all the steps of the HBV replication cycle subsequent to viral entry into the cell (6, 26, 34, 38, 41). Despite the relatively low efficiency of viral production in this system, this suggests that the transcriptional regulatory mechanisms which operate during infection of hepatocytes in vivo may be most precisely mimicked in these hepatoma cell lines. In addition, it is apparent that the *cis*-acting regulatory sequences of the nucleocapsid, large surface antigen, and X genes are located in regions of the HBV genome that code for a single polypeptide, whereas the regulatory sequences of the major surface antigen gene are located in a region of the genome where the pre-S and polymerase ORFs overlap (35). The requirements to conserve both of these ORFs such that they encode functional polypeptides presumably places restrictions on the nucleotide sequences which interact with the transcription factors involved in regulating the expression of the major surface antigen gene.

For these reasons, a detailed analysis of the transcriptional regulatory sequence elements which control the expression of the major surface antigen gene was performed by using transient transfection assays in the hepatoblastoma cell line HepG2. This analysis indicated that the role of the HBV enhancer in the regulation of the major surface antigen gene was limited to an approximately twofold increase in transcription. Sequence elements approximately 200 nucleotides immediately upstream of the transcription initiation sites were necessary for high-level expression from this promoter, whereas approximately 115 nucleotides were essential for transcriptional activity. Similar results were obtained with the hepatocellular carcinoma cell lines PLC/PRF/5 and Hep3B. The major surface antigen promoter elements defined in this study for HBV DNA (subtype ayw) differ from those previously identified for HBV DNA (subtype adw2) (9), suggesting possible subtype-specific regulation of this transcription unit.

MATERIALS AND METHODS

Plasmid constructions. The various steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (21). The HBV sequences in these constructions were derived from the plasmid pCP10, which contains two copies of the HBV genome (subtype ayw) cloned into the *Eco*RI site of pBR322 (12). The plasmid Sp (Fig. 1) was constructed by digesting pCP10 with *Xho*I, filling in the overhang with the Klenow fragment of *Escherichia coli* DNA polymerase, ligating *Hin*dIII linkers, digesting with *Hin*dIII, and cloning the 3.2-kilobase-pair HBV fragment into the *Hin*dIII site of the plasmid pUCCATpA (20). The unique HBV *Xho*I site used

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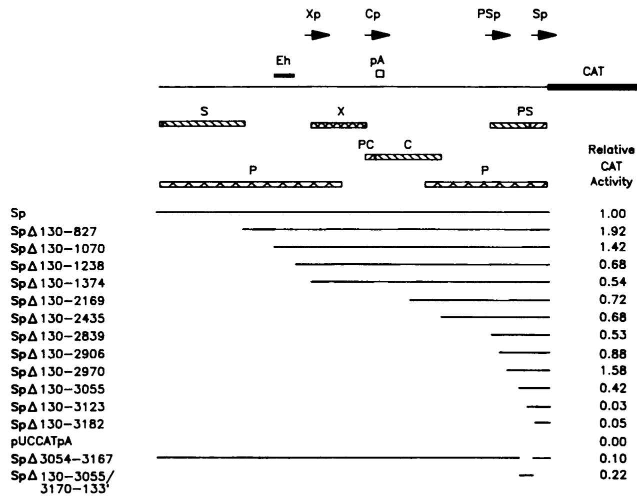

FIG. 1. Deletion analysis of the HBV major surface antigen promoter (SpΔ series) in HepG2 cells. Arrows indicate the positions and directions of transcription from the HBV X (Xp), core (Cp), pre-S1 (PSp), and surface (Sp) antigen promoters. Boxes indicate the positions of the HBV enhancer sequence (Eh), HBV polyadenylation sequence (pA), surface antigen ORF (S), X-gene ORF (X), presurface antigen ORF (PS), precore ORF (PC), core ORF (C), polymerase ORF (P), and chloramphenicol acetyltransferase ORF (CAT). The horizontal lines indicate the HBV sequences present in the various SpΔ series plasmids. The plasmid Sp contains the HBV sequences from nucleotides 130 to 3182/1 to 133' (nucleotide sequences are designated by using coordinates derived from the Genbank genetic sequence data bank). The designation 133' has been used to indicate that the nucleotides 130 to 133 are repeated twice in this plasmid and that nucleotides 130 to 133 and 130' to 133' are distal and proximal to the CAT ORF, respectively. The HBV sequences deleted from the various plasmids are designated by nucleotide coordinates. The pUC8 sequences in these plasmids are not shown.

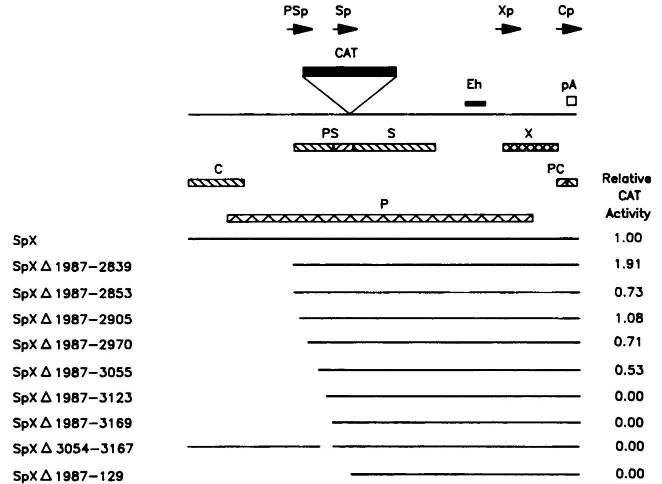

FIG. 2. Deletion analysis of the HBV surface antigen promoter (SpXΔ series) in HepG2 cells. Arrows, boxes, and line designations are as described for Fig. 1. The pUC13 sequences in these plasmids are not shown.

in this and subsequent constructs is located 157 nucleotides 3' to the surface antigen transcription initiation site (see Fig. 7). Therefore, the plasmid Sp contains one complete HBV genome located directly 5' to the promoterless chloramphenicol acetyltransferase (CAT) reporter gene such that the expression of the CAT gene is governed by the HBV major surface antigen promoter. The plasmid SpX (Fig. 2) was constructed by partially digesting pCP10 with *Bgl*II and cloning the 3.2-kilobase-pair HBV fragment beginning at nucleotide 1987 (as reported in Genbank genetic sequence data base) into the *Bam*HI site of pUC13, generating the plasmid pBg. Subsequently, the ends of the *Hin*dIII-*Bam*HI CAT gene fragment derived from pUCCATpA (20) were modified to *Xho*I sites by linker insertion, and the CAT gene was cloned into the *Xho*I site of pBg, generating the plasmid SpX. This plasmid contains the CAT reporter gene under the control of the HBV major surface antigen gene promoter. It differs from Sp in that the polyadenylation signal at the end of the CAT gene was not transferred into the SpX plasmid. Therefore, the only polyadenylation signal in this plasmid is derived from HBV sequences and presumably represents the signal utilized by the CAT transcripts. The plasmid constructs containing the various deletions, the SpΔ and SpXΔ series (Fig. 1 to 3), were generated by appropriate restriction endonuclease or *Bal* 31 nuclease digestions of HBV sequences and subsequent cloning steps similar to those described for Sp and SpX. All deletion breakpoints generated by *Bal* 31 nuclease digestion were determined by dideoxynucleotide sequencing (25). The extent of the deleted nucleotide sequence is indicated in the plasmid designation by using coordinates derived from the Genbank genetic sequence data bank.

Cells and transfections. The human hepatoblastoma cell line HepG2 and the human hepatocellular carcinoma cell

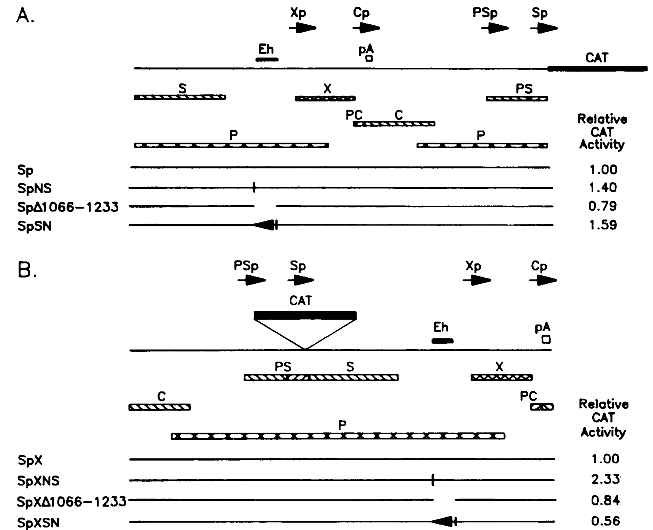

FIG. 3. Influence of the HBV enhancer sequence on the transcriptional activity of the major surface antigen promoter determined by using plasmids derived from Sp (A) and SpX (B) in HepG2 cells. Arrows, boxes, and line designations are as described for Fig. 1. Vertical lines indicate the locations where the *Nsi*I site in the HBV sequence was converted to an *Sph*I site by linker insertion to create the plasmids SpNS and SpXNS from the plasmids Sp and SpX, respectively. Elimination of the enhancer sequence was then performed by deletion of the 168-base-pair *Sph*I fragment from the plasmids SpNS and SpXNS to generate the plasmids SpΔ1066-1233 and SpXΔ1066-1233, respectively. Reintroduction of the *Sph*I enhancer fragment into SpΔ1066-1233 and SpXΔ1066-1233 in the reverse orientation relative to SpNS and SpXNS generated the plasmids SpSN and SpXSN, respectively.

lines PLC/PRF/5 (Alexander cells) and Hep3B were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum at 37°C in 5% CO_2–air and were transfected as previously described (22). The transfected DNA mixture comprised 15 μg of an Sp or SpX series plasmid and 1.5 μg of pSV2AL-AΔ5′ (10), which served as an internal control for transfection efficiency. pSV2AL-AΔ5′ directs the expression of the luciferase gene by using the simian virus 40 early promoter. Cell extracts were prepared 40 to 48 h after transfection and were assayed for luciferase and chloramphenicol acetyltransferase activity as previously reported (10).

RNA isolation and S1 nuclease analysis. RNA was isolated from transfected cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (8). The S1 nuclease protection analysis was performed by standard methods (21). RNA and 5′-end-labeled DNA were dissolved in 30 μl of 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl–1 mM EDTA, denatured at 80°C for 15 min, and annealed for 16 h at 48°C. Digestion of the hybridization mixture was performed by the addition of 300 μl of 50 mM sodium acetate (pH 4.6)–0.28 M NaCl–4.5 mM $ZnSO_4$ containing 20 μg of single-stranded calf thymus DNA per ml and 300 U of S1 nuclease and subsequent incubation for 30 min at 37°C. The digestion mixtures were precipitated with ethanol and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

DNase I footprinting. Nuclear extracts were prepared from HepG2 cells essentially as described previously (11). All operations were performed at 0 to 4°C. Cells were harvested from culture and centrifuged for 5 min at 2,000 rpm in a Sorvall RT6000 centrifuge. Pelleted cells were washed once in 20 ml of 10 mM sodium phosphate (pH 6.8)–0.14 M NaCl–1.5 mM $MgCl_2$ by suspension and recentrifugation for 5 min at 2,000 rpm. The cell pellet was suspended in 5 volumes of hypotonic buffer (10 mM Tris hydrochloride [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and allowed to stand for 10 min. Cells were collected by centrifugation for 5 min at 2,000 rpm, suspended in 2 volumes of hypotonic buffer, and lysed by 10 to 15 strokes of an all-glass Dounce homogenizer (B-type pestle). Cell lysis was more than 90%. The homogenate was centrifuged for 10 min at 3,000 rpm in a Sorvall SS34 rotor, and the supernatant was carefully poured off to leave a loose nuclear pellet. This was recentrifuged for 20 min at 15,000 rpm in the SS34 rotor, and the supernatant was decanted. The pellet was suspended in 2.5 volumes of nuclear extraction buffer (20 mM Tris hydrochloride [pH 7.9], 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and the nuclei were lysed with 10 strokes of the homogenizer. The lysate was stirred gently for 30 min and then centrifuged for 30 min at 15,000 rpm in the SS34 rotor. The supernatant was decanted and dialyzed for 5 h against 50 volumes of 20 mM Tris hydrochloride (pH 7.9)–20% (vol/vol) glycerol–100 mM KCl–0.2 mM EDTA–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride. The dialysate was clarified by centrifugation for 5 min at 14,000 rpm in a microcentrifuge, and the supernatant was frozen in aliquots in liquid N_2 and stored at −80°C. The DNase I footprinting reactions were performed as reported previously (2) and contained 1 to 5 ng of end-labeled DNA fragment in a 50-μl reaction mixture containing 25 mM Tris hydrochloride (pH 7.9), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCl, 10% glycerol, 1 μg of poly(dI) · poly(dC), and the nuclear extract to be assayed. Binding was carried out for 10 min at 0°C and then for 2 min at room temperature, and then 50 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ containing DNase I was added at room temperature. The quantity of DNase I used was dependent on the amount of nuclear extract in the assay. The reaction mixture was incubated at room temperature for 2 min and then stopped by the addition of 100 μl of 1% (wt/vol) sodium dodecyl sulfate–20 mM EDTA–200 mM NaCl containing 250 μg of tRNA per ml. The mixture was then extracted with phenol, precipitated with ethanol, and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

RESULTS

Deletion analysis of the HBV major surface antigen gene promoter. The analysis of the transcriptional regulatory elements that control the expression of the HBV major surface antigen gene was performed by using transient expression assays in the human hepatoblastoma cell line HepG2. Variations in transfection efficiencies were normalized by determining the percentage of chloramphenicol which was acetylated by the CAT activity per 100,000 light units produced by the luciferase activity in the same cell extract. The relative CAT activity was then determined by calculating the ratio of the normalized CAT activity observed after transfection of plasmids in the SpΔ and SpXΔ series to the normalized CAT activity observed after transfection of plasmids Sp and SpX, respectively. The expression of the CAT reporter gene was placed under the control of the surface antigen promoter, which in the initial plasmid, Sp (Fig. 1), comprised the complete sequence of the HBV genome. A series of 5′ deletions of the HBV genome were tested for their effects on the transcriptional activity of the surface antigen promoter (Fig. 1). Transcriptional activity was not lost when sequences from nucleotides 130 to 3055 were deleted, indicating that a minimal promoter element exists between nucleotides 3056 and 133′. In addition, the plasmid SpΔ130-3055/3170-133′ showed only a twofold reduction in relative CAT activity compared with the plasmid SpΔ130-3055, indicating that the minimal promoter element is present between nucleotides 3056 and 3169. The difference in relative CAT activity between these two constructs is probably not due to the deletion of promoter sequences but results from the deletion of 146 nucleotides (nucleotides 3170 to 133′) which are transcribed into the CAT RNA coded for by the plasmid SpΔ130-3055 but have been deleted from the CAT transcript expressed from the plasmid SpΔ130-3055/3170-133′.This additional deletion shortens the 5′ untranslated region of this transcript, which may influence its stability or efficiency of translation. Therefore, the minimal promoter element is located between −103 and +11 relative to a predominant transcriptional initiation site (see Fig. 7) identified at approximately nucleotide 3159 (Fig. 4). Further evidence demonstrating the essential role of these sequences in regulating the activity of the surface antigen promoter was obtained by determining the relative CAT activity derived from the plasmid SpΔ3054-3167. In this case, deletion of 114 nucleotides resulted in the loss of 90% of the relative CAT activity. In addition to the minimal promoter element, it appears that the sequences between nucleotides 2971 and 3055 (−188 and −104 relative to the transcriptional initiation site) may influence the activity of the surface antigen promoter, as there was approximately a fourfold reduction in relative CAT activity between the plasmids SpΔ130-2970 and SpΔ130-3055 (Fig. 1).

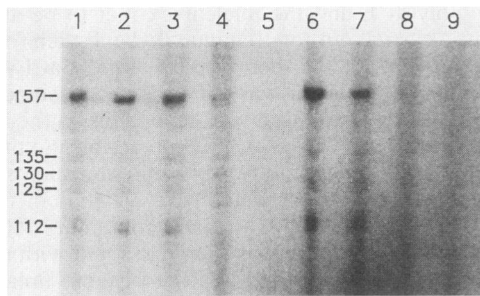

FIG. 4. S1 nuclease analysis of the transcription initiation sites for the HBV major surface antigen promoter. RNA (25 μg) from HepG2 cells transfected with Sp (lane 1), SpΔ130-1238 (lane 2), SpΔ130-3055 (lane 3), SpΔ130-3123 (lane 4), pUCCATpA (lane 5), SpX (lane 6), SpXΔ1987-3055 (lane 7), SpXΔ1987-3123 (lane 8), and SpXΔ1987-129 (lane 9) was hybridized to the 476-nucleotide HBV *Bgl*II-*Xho*I fragment (nucleotides 2840 to 133) which was 5'-end labeled at the *Xho*I site. After digestion with S1 nuclease, the protected fragments were resolved in 6% urea–acrylamide sequencing gels and subjected to autoradiography.

The role of the HBV enhancer sequence in the regulation of the expression from the surface antigen promoter appears to be limited. There was only a twofold difference in relative CAT activity between the transcriptional activity of the plasmids SpΔ130-1070 and SpΔ130-1238, which differ only by a 138-nucleotide sequence which includes the HBV enhancer sequence (29). Interpretation of this result has been complicated by the recent observation that the product of the X gene can transactivate the simian virus 40 early promoter in HepG2 cells (39). Measurements of the absolute amounts of luciferase activity produced by cotransfection of pSV2AL-AΔ5' with plasmids of the SpΔ series suggest that transactivation of the simian virus 40 early promoter by the X-gene product, if it occurs in our system, is limited to only a two- to fourfold increase in the presence of plasmids Sp through SpΔ130-1374 (data not shown). This modest effect does not significantly alter the interpretation of this deletion analysis. In addition, experiments using the mouse metallothionein promoter to transcribe the luciferase gene as an internal control produced results similar to those using pSV2AL-AΔ5' (data not shown).

The sequence elements that regulate the expression of the surface antigen gene were further characterized by analyzing the transcriptional activity of the SpX series of plasmids (Fig. 2). These deletions differ from the Sp series of plasmids in that the CAT gene has been inserted into the surface antigen transcription unit such that CAT should be encoded by an RNA which is identical to the 2.1-kilobase transcript produced during infection except that it contains the CAT ORF inserted 28 nucleotides 5' to the major surface antigen initiation codon. In addition, the complete series of constructs contains the X gene and the regulatory sequences necessary for its expression (31, 37). This deletion analysis revealed that the same minimal promoter element located within 103 nucleotides 5' to the transcriptional initiation site was essential for promoter activity (SpXΔ1987-3055).

The contribution of the HBV enhancer sequence to the transcriptional activity from the surface antigen promoter was further characterized by deleting this sequence from the Sp and SpX plasmids (Fig. 3). The plasmids SpΔ1066-1233 and SpXΔ1066-1233 showed a less than threefold reduction in relative CAT activity, confirming that deletion of the enhancer in this system had very little influence on the transcriptional activity of the surface antigen promoter. In addition, the result of deletion of the enhancer sequence in the plasmid SpXΔ1066-1233 also indicates that this sequence does not appear to play a major role in determining the stability of the HBV 2.1-kilobase transcript, as might have been suggested from previous results (40). Reintroduction of the enhancer sequence into the SpΔ1066-1233 plasmid in the reverse orientation relative to SpNS, generating SpSN, resulted in an approximately twofold increase in transcriptional activity from the surface antigen promoter, demonstrating that this sequence can display the properties of a very weak enhancer. However, reintroduction of this sequence into SpXΔ1066-1233 in a similar manner to generate the plasmid SpXSN produced essentially no change in the relative CAT activity. This is probably the result of the inclusion of this antisense enhancer sequence into the CAT RNA which may have some influence on the stability or efficiency of translation of this transcript.

Characterization of the major surface antigen gene transcription initiation sites. The transcriptional activities of the various surface antigen promoter plasmids were determined indirectly by utilizing the CAT reporter gene (Fig. 1 to 3). Direct determination of the abundance of correctly initiating transcripts was performed by S1 nuclease protection analysis of RNA isolated from HepG2 cells transfected with various plasmids to confirm these observations (Fig. 4). It is apparent that a predominant transcription initiation site mapping to nucleotide 3159 (157 nucleotides from the *Xho*I site at nucleotide 133) was utilized in these transient transfection assays (Fig. 4; see Fig. 7). Several additional minor transcription initiation sites mapping approximately 112, 125, 130, and 135 nucleotides (nucleotides 22, 9, 4, and 3181, respectively) from the *Xho*I site were also observed. These initiation sites are in good agreement with previous reports (27, 41). Correctly initiating transcripts were only observed in RNA isolated from cells transfected with constructs containing the minimal promoter element located between −103 and +11. These results also confirm that sequences 5' to −103, including the enhancer element, appear to be nonessential for significant levels of correct transcriptional initiation from the major surface antigen promoter.

Identification of nuclear factors which bind the major surface antigen promoter region. The location where nuclear factors extracted from HepG2 cells bind to the major surface antigen promoter was examined by DNase I footprinting. Distinct protection of four regions (IL, IIL, IIIL, and IVL) on the long (L) or minus strand and five regions (IS, IIS, IIISa, IIISb, and IVS) on the short (S) or plus strand of the HBV DNA was observed (Fig. 5). The positions of these protected regions were accurately determined by comparison with the sequencing reaction products of an unrelated DNA of known sequence resolved on the same gel. The positions of these regions which were protected from DNase I digestion by factors present in the HepG2 nuclear extracts were compared with the locations of the various promoter deletion breakpoints which influence the transcriptional activity of the surface antigen promoter in the transient transfection assays (see Fig. 7).

It is apparent that the factor(s) responsible for footprint region I binds DNA sequences very close to the deletion breakpoint at nucleotide 2970. This region contains the consensus sequence for the transcription factor NF1 (nucleotides 2970 to 2983) which has previously been shown to bind to this sequence in the HBV genome (28). It therefore seems likely that NF1 or a related transcription factor is responsible for binding to region I. The factor(s) binding to footprint region II appear to bind to sequence elements

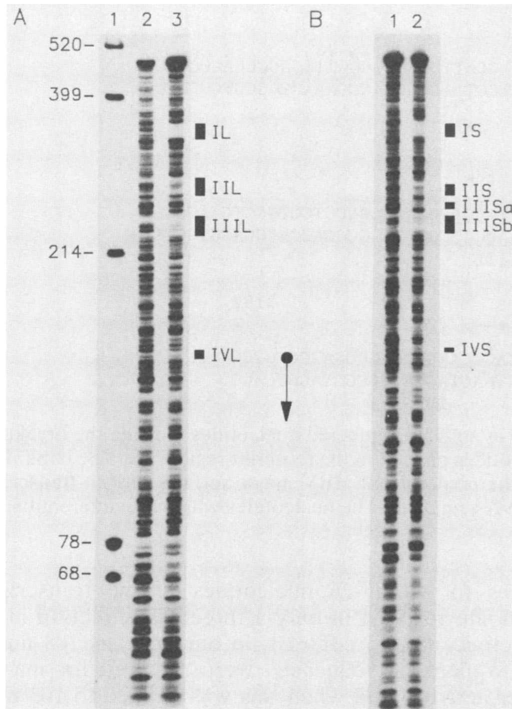

FIG. 5. DNase I footprinting analysis of the HBV major surface antigen promoter. The 476-nucleotide HBV *Bgl*II-*Xho*I DNA fragment (nucleotides 2840 to 133) was 5′ end labeled (A) or 3′ end labeled (B) at the *Xho*I site, and 5 ng was incubated in the absence of (lanes A2 and B1) or in the presence of 30 (lane A3) or 70 μg (lane B2) of HepG2 nuclear extract before DNase I digestion. Lane A1, pUC13 digested with *Hin*fI. Regions protected from DNase I digestion were indicated (■) and designated IL, IIL, IIIL, IVL, IS, IIS, IIISa, IIISb, and IVS. Arrow indicates the approximate locations of the major surface antigen gene RNA initiation sites and the direction of transcription.

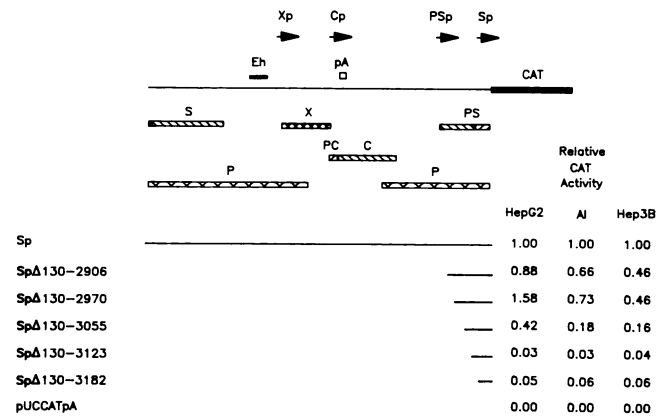

FIG. 6. Deletion analysis of the HBV surface antigen promoter (SpΔ series) in HepG2, PLC/PRF/5 (Al), and Hep3B cells. Arrows, boxes, and line designations are as described for Fig. 1.

which are 5′ to the deletion breakpoint at 3055. This therefore suggests that the transcription factors which interact with regions I and II are not essential for transcriptional activity from the surface antigen gene but they may be involved in influencing its activity. Footprint region III comprises two protected regions on the short strand, IIISa and IIISb, and one protected region on the long strand, IIIL. It should be noted that the separation of the protected regions between nucleotides 3032 and 3091 into footprint regions II and III is based on the minimal essential promoter region being functionally defined between nucleotides 3056 and 3169 (Fig. 1). These designations are not meant to indicate the location of regions protected from DNase I digestion by specific individual transcription factors. The minimal essential surface antigen gene promoter region also includes footprint region IV, which is located at the extreme end of the 5′ untranslated region of the longest surface antigen transcript. The factors which bind to regions III and IV are likely to be involved in regulating the transcriptional activity of the surface antigen promoter.

Characterization of the HBV major surface antigen promoter sequences in differentiated hepatocellular carcinoma cell lines. The transcriptional regulatory sequence elements involved in controlling the level of expression of the HBV major surface antigen promoter in the hepatocellular carcinoma cell lines PLC/PRF/5 and Hep3B were determined (Fig. 6). The results indicate that sequences between nucleotides 2971 and 3055 influence the level of transcription from the surface antigen promoter, increasing the activity three- to fourfold. In addition, sequences within 103 nucleotides of the transcriptional initiation site were essential for minimal promoter activity (plasmid SpΔ130-3055). These results indicate that the same sequences are involved in the regulation of the HBV major surface antigen gene in the three differentiated hepatoma cell lines examined.

DISCUSSION

The HBV genome (subtype ayw) comprises 3,182 nucleotides and contains all the information necessary for the production of infectious virions after its introduction into the differentiated hepatoblastoma cell line HepG2 by transfection (33, 34). The small size of the genome and the observation that every nucleotide resides in at least one ORF suggests that the sequence elements which regulate the expression of the HBV genes might possess limited complexity. In an attempt to examine this possibility, the regulatory sequence elements which are present in the complete HBV genome and which influence the transcriptional activity of the major surface antigen gene promoter in differentiated hepatoma cell lines were characterized.

In HepG2 cells, it was apparent that the HBV enhancer had little effect on the transcriptional activity of the major surface antigen promoter. This was the case whether the enhancer sequence was located 5′ to or within the CAT transcription unit (Fig. 1 and 3). In each case, the position of the enhancer sequence corresponded to its normal location in the HBV genome. The influence of the enhancer sequence on the activity of the surface antigen gene promoter has been investigated in a variety of different systems (1, 3, 7, 14). In two cases (1, 7), the presence of the enhancer resulted in increased transcriptional activity from the surface antigen promoter by 20- to 60-fold in the various hepatoma cell lines used in these studies. However, these results were obtained by moving the enhancer sequence from its normal position in the HBV genome. The relocation of the enhancer may increase its ability to influence the transcriptional activity of the surface antigen promoter. Consistent with this possibility, deletion of the enhancer sequence from the surface antigen transcription unit has been shown to result in a very modest decrease in transcriptional activity from the surface

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S: CAATCCCAACAAGGACACCTGGCCAGACGCCAACAAGGTAGGAGCTGGAGCATTCGGGCTGGGTTTCACCCCACCGCACG
L: GTTAGGGTTGTTCCTGTGGACCGGTCTGCGGTTGTTCCATCCTCGACCTCGTAAGCCCGACCCAAAGTGGGGTGGCGTGC

GAGGCCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCATACTACAAACTTTGCCAGCAAATCCGCCTCCTGCCTCCACCAAT
CTCCGGAAAACCCCACCTCGGGAGTCCGAGTCCCGTATGATGTTTGAAACGGTCGTTTAGGCGGAGGACGGAGGTGGTTA

CGCCAGTCAGGAAGGCAGCCTACCCCGCTGTCTCCACCTTTGAGAAACACTCATCCTCAGGCCATGCAGTGG
GCGGTCAGTCCTTCCGTCGGATGGGGCGACAGAGGTGGAAACTCTTTGTGAGTAGGAGTCCGGTACGTCACC
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Labels on the figure: 2970, IS, IL (line 1); 3055, IIS, IIISa, IIISb, IIL, IIIL (line 2); 3123, •—> IVS, 3169, 3182, IVL (line 3).

FIG. 7. Sequence of the HBV major surface antigen promoter region (subtype ayw). The numbered nucleotides indicate the breakpoints of functionally significant deletions. The underlined sequences represent the nucleotides present in the footprint regions IS, IIS, IIISa, IIISb, IVS, IL, IIL, IIIL, and IVL. The arrow indicates the approximate location of the predominant HBV major surface antigen transcription initiation site. S, Short or plus-strand DNA sequence; L, long or minus-strand DNA sequence. The nucleotides which are different between the ayw and adw2 sequences are indicated (*).

antigen promoter in HepG2 cells (3). It has also been suggested that the enhancer may increase transcription from the surface antigen promoter in a cell-specific manner (14). The activity of the surface antigen promoter in the hepatoma cell line PLC/PRF/5 was shown to be independent of the enhancer sequence, whereas the enhancer sequence increased transcription from this promoter approximately 30-fold in the hepatoma cell line SK-Hepl (14). Our results are compatible with the suggestion that the enhancer sequence, when located at its normal location in the HBV genome, may only slightly modulate the level of expression from the surface antigen promoter in HepG2 cells. In addition, it is apparent that transcriptional activity from the other HBV promoters does not significantly influence the transcriptional activity from the surface antigen promoter (Fig. 1 and 2).

The detailed deletion analysis of the HBV major surface antigen promoter indicated that correctly initiated transcription required only 103 nucleotides of 5′-flanking sequences. However, an additional 85 nucleotides (−104 to −188 relative to the transcription initiation site) of 5′-flanking sequence immediately adjacent to this element increased the transcriptional activity of the surface antigen promoter approximately fourfold (Fig. 1, 6, and 7). These results obtained from transient transfection assays define a regulatory region which was shown to bind several nuclear factors isolated from HepG2 cells (Fig. 5). The footprint region I contains the consensus sequence TGG$(N)_{6-7}$GCCAA, which has been shown to bind the transcription factor NF1 (28). Since all but the first nucleotide of this consensus sequence is retained in the plasmid SpΔ130-2970, it is possible that this or a related factor is involved in modulating the transcriptional activity of the surface antigen promoter. It is also likely that the factor(s) responsible for footprint region II is also involved in the modulation of the activity of the surface antigen promoter, whereas the factors responsible for footprint regions III and IV may represent essential transcription factors, since they bind the minimal promoter region −103 to +11 (Fig. 7).

The promoter elements defined in this analysis differ significantly from the surface antigen regulatory sequences defined previously in PLC/PRF/5 cells (9). In the previous report (9), only 81 nucleotides 5′ to the transcription initiation site (nucleotide 3159 [Fig. 7]) were essential for maximum activity of the surface antigen promoter. Deletion of sequences to within 26 nucleotides of the transcription initiation site resulted in only a threefold reduction in promoter activity (9). In contrast, in our analysis 188 nucleotides of 5′-flanking sequences were required for maximal promoter activity, and when this was deleted to 103 nucleotides there was a fourfold reduction in activity. Activity was completely lost when the 5′-flanking sequence was deleted to 36 nucleotides. These observations suggest these two HBV surface antigen promoters might be regulated differently. Since this analysis was performed with HBV DNA (subtype ayw) and the previous analysis used HBV DNA (subtype adw2), it is possible that different subtypes of HBV have evolved to the extent where they now possess distinct mechanisms of gene regulation. Comparison of the HBV_{ayw} and HBV_{adw2} sequences in the surface antigen promoter region reveals nucleotide differences in footprint regions I to III, suggesting the possibility that transcription factors might distinguish between these subtype-specific sequence elements. A more detailed analysis of the sequences involved in the regulation of the surface antigen promoter of these two HBV subtypes will be required to resolve this issue.

ACKNOWLEDGMENTS

We are grateful to Hung Fan (University of California, Irvine) for plasmid pUCCATpA, Donald Helinski (University of California, San Diego) for plasmid pSV2AL-AΔ5′, and Pierre Tiollais (Institut Pasteur, Paris, France) for plasmid pCP10. We thank Lisa Podhajsky for preparation of the manuscript.

This work was supported by Public Health Service grants AI00585, AI20720, and AI25183 from the National Institutes of Health.

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