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Regulation of Transcription from the Hepatitis B Virus Major Surface Antigen Promoter by the Sp1 Transcription Factor†

ANNEKE K. RANEY, HIEN B. LE, AND ALAN McLACHLAN*

Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

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The DNA-binding proteins which recognize the regulatory sequence elements of the hepatitis B virus (HBV) major surface antigen promoter were examined by gel retardation analysis, using nuclear extracts from the human hepatoma cell line Huh7. Using this assay, we identified four regions (B, D, E, and F) of the promoter that interact with the same or similar transcription factor(s). In addition, the recognition sequence for the Sp1 transcription factor bound the same or similar transcription factor(s) present in Huh7 cell nuclear extracts, and this binding was inhibited by the four major surface antigen promoter elements, B, D, E, and F. Purified Sp1 transcription factor was shown to bind to three (B, D, and F) of the major surface antigen promoter regulatory sequence elements by DNase I footprinting. Using transient transfection assays with *Drosophila* Schneider line 2 cells, we found that transcription from the major surface antigen promoter was transactivated by exogenously expressed Sp1, whereas transcription from the other three HBV promoters was not. Deletion analysis of the major surface antigen promoter demonstrated that the promoter region between −35 and +157 was sufficient to confer Sp1 responsiveness. This promoter region includes one of the regulatory elements footprinted by the purified Sp1 transcription factor. The function of the B, D, E, and F promoter elements was further examined by using these binding sites cloned into a minimal promoter element. Each of these regulatory regions transactivated transcription from the minimal promoter element in response to exogenously expressed Sp1. This finding demonstrates that the HBV major surface antigen promoter contains four functional Sp1 binding sites which probably contribute to the level of expression from this promoter during viral infection.

During hepatitis B virus (HBV) infection, the 3.2-kbp partially double-stranded circular viral DNA genome is converted to a covalently closed relaxed circular double-stranded conformation which is the presumed template for transcription (64). The HBV genome is transcribed by the host's cellular transcriptional machinery to produce transcripts of 3.5, 2.4, 2.1, and 0.7 kb (26). These transcripts are translated into the nucleocapsid and polymerase polypeptides, the large surface antigen polypeptide, the middle and major surface antigen polypeptides, and the X-gene polypeptide (11, 12, 26, 29, 31, 53, 68). The surface antigen and nucleocapsid polypeptides represent the major HBV structural proteins. The polymerase polypeptide is incorporated into the immature core particle, where it converts the 3.5-kb pregenomic RNA into the partially double stranded circular viral genomic DNA (4, 27, 36). The X-gene product appears to encode a polypeptide capable of transactivating transcription from a variety of viral and cellular promoters (15, 46, 49, 52, 57–60, 65, 71).

The regulatory sequence elements which control the synthesis of the HBV transcripts have been characterized in a variety of cell lines (14, 17, 28, 32, 33, 35, 38, 39, 42–44, 47, 50, 51, 55, 62, 66, 67). Two transcriptional enhancer elements that appear to influence the level of transcription from the four viral promoters have been identified (1, 10, 13, 22, 23, 30, 34, 48, 54, 61, 63, 69, 70, 72). The transcription factors which bind to the enhancer and promoter sequences and regulate the level of RNA synthesis from the viral promoters have not been extensively characterized. The importance of hepatocyte nuclear factor 1 (HNF1) in the regulation of expression from the large surface antigen promoter has been reported, and the CCAAT/enhancer-binding protein appears to modulate HBV transcriptional activity from both enhancer 1 sequence elements and the nucleocapsid promoter (20, 33, 34, 37, 39, 56). The observation that both HNF1 and the CCAAT/enhancer-binding protein are liver-enriched transcription factors (5–7, 25) may account, in part, for the highly tissue-specific expression of HBV during infection and in transgenic mice (2, 24, 41).

Previously, it had been shown by functional analysis that the level of transcription from the major surface antigen promoter is controlled by the complex interplay between a minimum of six transcription factors which activate and one transcription factor which represses transcription from this promoter (44). However, the identities of the transcription factors which mediate this regulation were not examined. In this study, the transcription factors which bind to the regulatory sequence elements of the major surface antigen promoter were characterized to examine their roles in the regulation of transcription from this promoter. This analysis demonstrated that there are four functional binding sites for the Sp1 transcription factor located in the previously identified regulatory regions B, D, E, and F of the major surface antigen promoter (44).

MATERIALS AND METHODS

Plasmid constructions. The plasmid constructs used in the transfection experiments were cloned by standard techniques (45). The HBV sequences in these constructions were derived from plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the *Eco*RI site of pBR322 (21). Plasmid SpLUC has been described previously (42). The unique HBV *Xho*I site used in this construct is

* Corresponding author.
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located 157 nucleotides 3′ to a predominant surface antigen gene transcription initiation site (11, 43). Therefore, plasmid SpLUC contains one complete HBV genome located directly 5′ to the promoterless firefly luciferase reporter gene such that expression of this gene is governed by the HBV major surface antigen promoter. Similarly, plasmids XpLUC, CpLUC, and PS(1)pLUC, containing one complete HBV genome, were constructed such that expression of the luciferase gene is governed by the hepatitis B X-gene, nucleocapsid, and large surface antigen promoters, respectively. Details of the construction of these plasmids and of the various 5′ deletions and linker scanning mutations in the major surface antigen promoter have been described previously (42, 44). Plasmid Δ5′−188 contains the major surface antigen promoter region from −188 (coordinate 2971) to +157 (coordinate 133). A predominant transcription initiation site previously observed in transient transfection assays (43) has been designated +1 (coordinate 3159). The coordinates of the major surface antigen promoter region are derived from the GenBank genetic sequence data bank.

Plasmid TATALUC was constructed by cloning a double-stranded oligonucleotide containing the large surface antigen promoter TATA-box sequence (coordinates 2773 to 2791), produced by annealing the oligonucleotides CTATATTATA TAAGAGAGAAGCT and TCTCTCTTATATAATATAG GTAC, into the *Sac*I and *Kpn*I sites of plasmid p19DLUC (42). Similarly, oligonucleotides A to G and Sp1 (Fig. 1 and 7) containing TATALUC series plasmids were constructed by cloning double-stranded oligonucleotides into the unique *Sal*I site of plasmid TATALUC. The oligonucleotide pairs used to generate these double-stranded oligonucleotides were TCGATGGCCAGACGCCAACA and TCGATGTTG GCGTCTGGCCA (oligonucleotide A, coordinates 2970 to 2985), TCGACCTTTTGGGGTGGAGCCCTC and TCGAG AGGGCTCCACCCCAAAAGG (oligonucleotide B, coordinates 3035 to 3054), TCGAGGCTCAGGGCATACTACAAA CTTTGCCAGCAAATCC and TCGAGGATTTGCTGGCA AAGTTTGTAGTATGCCCTGAGCC (oligonucleotide C, coordinates 3055 to 3091), TCGAAATCCGCCTCCTGCC and TCGAGGCAGGAGGCGGATT (oligonucleotide D5′, coordinates 3086 to 3103), TCGAGCCTCCTGCCTCCAC-CAATC and TCGAGATTGGTGGAGGCAGGAGGC (oligonucleotide D, coordinates 3092 to 3111), TCGAAGTCAG GAAGGCAGCCTACCCC and TCGAGGGGTAGGCTGCC TTCCTGACT (oligonucleotide E, coordinates 3115 to 3136), TCGAAGCCTACCCCGCTGTCTCCACCTT and TCGAA AGGTGGAGACAGCGGGGTAGGCT (oligonucleotide F, coordinates 3127 to 3151), TCGAGCTGTCTCCACCTT and TCGAAAGGTGGAGACAGC (oligonucleotide F3′, coordinates 3137 to 3151), TCGATGAGAAACACTCATCCTCAG GCCA and TCGATGGCCTGAGGATGAGTGTTTCTCA (oligonucleotide G, coordinates 3151 to 3175), and TCGAGG GGCGGGGC and TCGAGCCCCGCCCC (oligonucleotide Sp1 [9]).

Cells and transfections. The human hepatoma cell line Huh7 was grown in RPMI 1640 medium and 10% fetal calf serum at 37°C in 5% CO_2–air. The *Drosophila* Schneider line 2 (SL2) cells were grown in Schneider's *Drosophila* medium (GIBCO Laboratories) containing 10% heat-inactivated fetal calf serum at 25°C. The SL2 cells were transfected as described previously (16) with a DNA mixture consisting of 1 μg of a luciferase plasmid, 200 ng of the Sp1 expression vector pPacSp1 (16) or 200 ng of the control expression vector pPacU (16), and 10 ng of p5CCAT (8). p5CCAT directs the expression of the chloramphenicol acetyltransferase gene under the control of the *Drosophila* actin 5C promoter and served as an internal control for transfection efficiency. Cell extracts were prepared 40 to 48 h after transfection and assayed for luciferase and chloramphenicol acetyltransferase activities as previously reported (18, 42, 43).

Nuclear extracts, gel retardation analysis, and DNase I footprinting. Nuclear extracts were prepared from Huh7 cells essentially as described previously (19). 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 [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) 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 material was recentrifuged for 20 min at 15,000 rpm in the SS34 rotor, and the supernatant was decanted. The pellet was resuspended 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 DTT, 0.5 mM PMSF), 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 DTT–0.5 mM PMSF. 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.

Gel retardation analysis was performed as described previously (39). Samples (1 ng) of ^{32}P-labelled double-stranded oligonucleotides representing regulatory regions (A to G) of the major surface antigen promoter or an Sp1 recognition site (see above for plasmid constructions) were incubated with 9 μg of nuclear extract prior to 4% polyacrylamide gel electrophoresis and autoradiography (3). When gel retardation competition analysis was performed, the nuclear extract was preincubated with 1 μg of cold double-stranded competitor oligonucleotide for 15 min prior to the addition of the ^{32}P-labelled double-stranded oligonucleotides.

The DNase I footprinting reactions were performed as reported previously (9, 43), using 1 to 5 ng of end-labelled 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 DTT, 50 mM KCl, 10% glycerol, and the purified Sp1 transcription factor (Promega) to be assayed. Binding was carried out for 15 min at 0°C and then for 2 min at room temperature, after which 50 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ containing DNase I was added at room temperature. The reaction mixture was incubated at room temperature for 2 min, and the reaction was stopped by the addition of 100 μl of 1% (wt/vol) SDS–20 mM EDTA–200 mM NaCl containing 250 μg of tRNA per ml. The mixture was then precipitated with ethanol and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

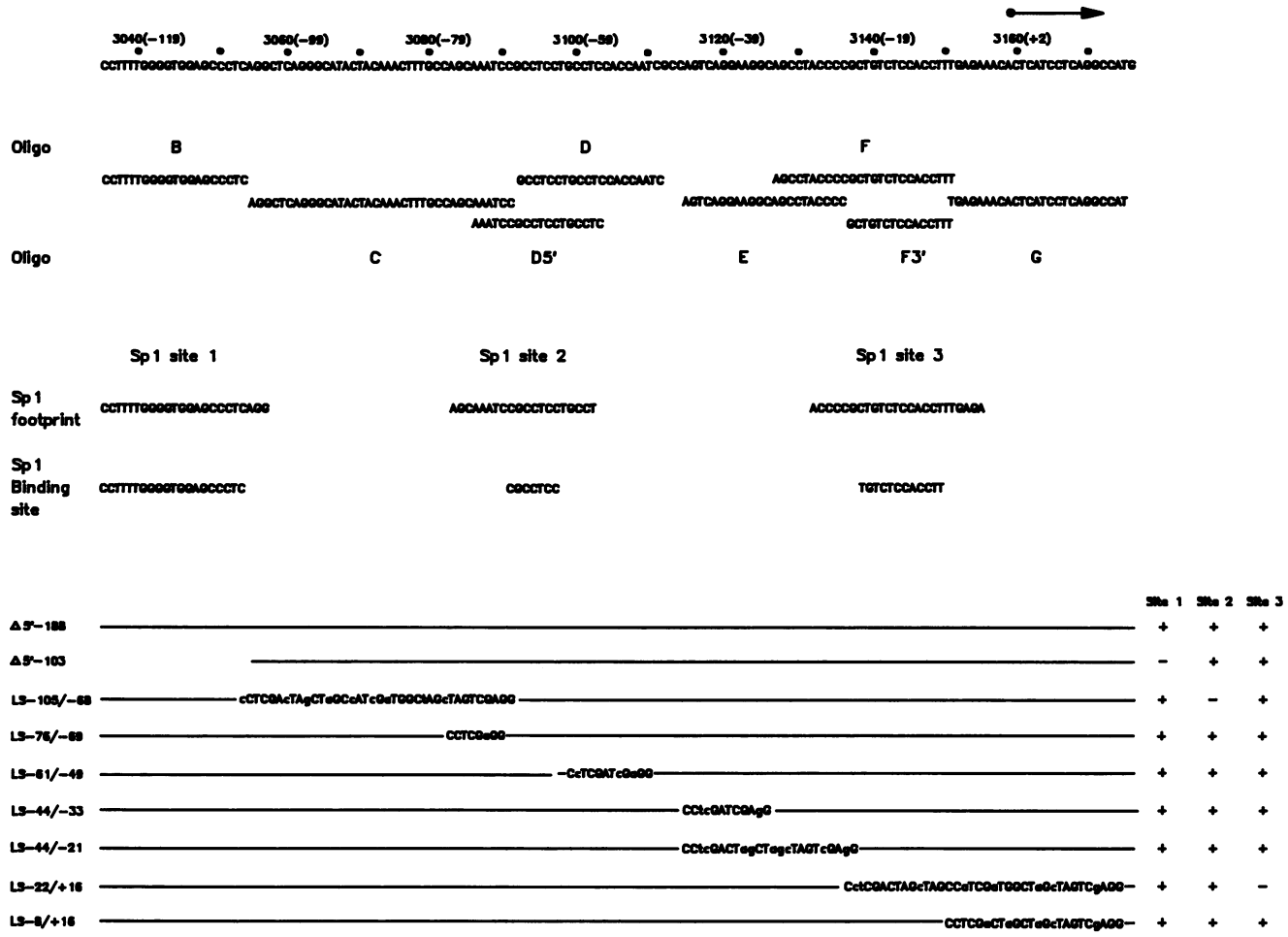

FIG. 1. Sequences of the HBV major surface antigen promoter (top sequence), oligonucleotides B to G, Sp1 footprint sites 1 to 3 defined by DNase I digestion of the wild-type promoter (Fig. 4 and 5), the essential binding sites for Sp1 sites 1 to 3 defined by DNase I footprinting of linker scanning mutants of the major surface antigen promoter (Fig. 5). Also shown are the sequences of the linker scanning mutations and the effects that they demonstrated on the DNase I footprinting pattern. The promoter sequence (subtype *ayw*) is shown with nucleotide coordinates and transcription initiation site (arrow) indicated. The coordinates of the major surface antigen promoter region are derived from the GenBank genetic sequence data bank, and their positions relative to a predominant transcription initiation site (+1, nucleotide coordinate 3159) are given in parentheses. The linker sequences of the linker scanning mutants are shown; nucleotide substitutions are indicated in uppercase, whereas unchanged nucleotides are indicated in lowercase. Deletion of a nucleotide is indicated by a hyphen. The linker scanning mutations were introduced into construct Δ5′−188 (44).

RESULTS

Characterization of the DNA-binding proteins which interact with the HBV major surface antigen gene promoter by gel retardation analysis. It has been shown previously that the HBV major surface antigen gene promoter comprises a minimum of seven functional regions (A to G) which are involved in regulation of the level of expression of the 2.1-kb surface antigen mRNA (44). In an attempt to characterize further the mechanism of transcriptional regulation of this promoter, the DNA-binding proteins present in Huh7 cell nuclear extracts were examined for the ability to interact with the functional regions of the major surface antigen promoter. Initially, double-stranded oligonucleotides representing the functional promoter regions A to G (Fig. 1) were examined for the ability to bind factors present in Huh7 cell nuclear extracts. This analysis demonstrated that each of the seven double-stranded oligonucleotides bound specifically to a factor(s) in the nuclear extract, since the corresponding cold double-stranded oligonucleotide inhibited the formation of the gel retardation complexes (Fig. 2). In addition, their migration properties indicated that the gel retardation complexes observed with oligonucleotides B, D, E, and F might be the same or similar (40).

To investigate the possibility that the same or similar factors bind to the major surface antigen regulatory region double-stranded oligonucleotides, the ability of each of the double-stranded oligonucleotides (A to G) to inhibit the formation of each of the observed complexes was examined (Fig. 2). When ^{32}P-labelled double-stranded oligonucleotide C was examined, the retardation complex which formed was inhibited only when double-stranded oligonucleotide C was used as a competitor. This result is consistent with a distinct DNA-binding protein(s) binding to region C and to no other examined region of the major surface antigen promoter. Similar results were obtained for ^{32}P-labelled double-stranded oligonucleotide G. When ^{32}P-labelled double-

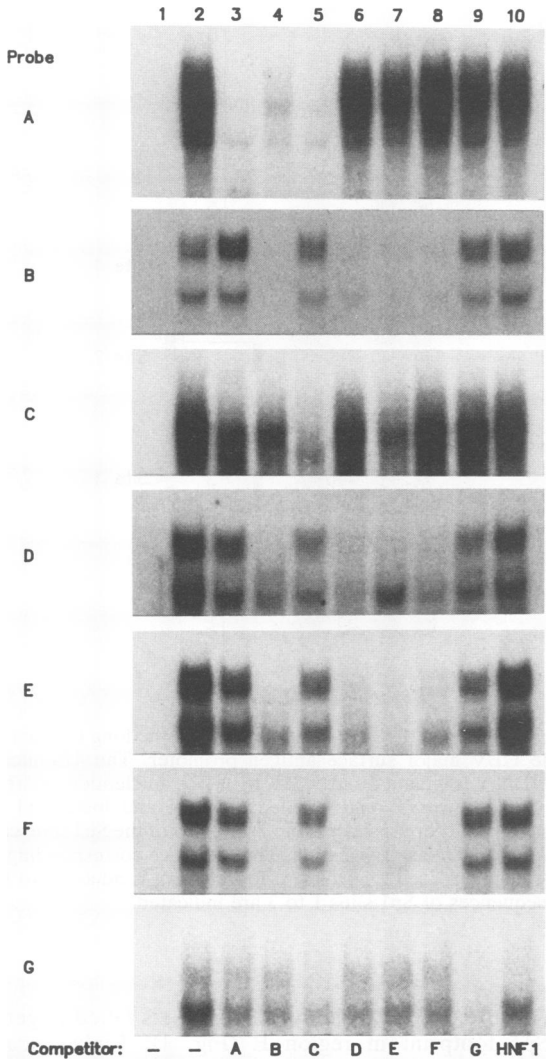

FIG. 2. Gel retardation and complex inhibition analysis of the major surface antigen promoter regulatory regions. ^{32}P-labelled double-stranded oligonucleotide probes A to G were analyzed for the ability to form complexes with DNA-binding proteins present in Huh7 cell nuclear extracts. Unlabelled double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. An HNF1 double-stranded oligonucleotide binding site was included as a nonspecific competitor control. Only the complexes that were formed are shown. Nuclear extract was omitted from lane 1.

stranded oligonucleotide A was examined, the retardation complex(es) which formed was inhibited when double-stranded oligonucleotides A, B, and C were used as competitors. This result was not anticipated, as the retardation complex(es) formed with the use of oligonucleotide A was different in mobility from those observed with oligonucleotides B and C (40). These inhibition results were also not reciprocal (Fig. 2), indicating that although regions B and C might compete for the factor(s) binding to oligonucleotide A when they are present in large molar excess, they preferentially bind different factors when examined in limiting amounts in the Huh7 nuclear extract. The significance of this observation with regard to control of the level of expression from the major surface antigen promoter is currently unclear. However, previous characterization of the factor(s) binding to region A has demonstrated that this regulatory element binds nuclear factor 1 (47).

When the ^{32}P-labelled double-stranded oligonucleotides B, D, E, and F were examined, the formation of retardation complexes was inhibited (Fig. 2). This effect was clearly apparent for the slower-migrating complex. However, this result was less obvious for the faster-migrating complex in the case of ^{32}P-labelled double-stranded oligonucleotides D and E. Since the inhibition results were reciprocal, it appears that these four regions of the major surface antigen promoter bind the same or similar factor(s).

The observation that oligonucleotides E and F can bind the same factor(s) would not be surprising if the recognition sequence were located in the region of overlap between these two oligonucleotides (Fig. 1). To examine this possibility, an additional ^{32}P-labelled double-stranded oligonucleotide, F3′ (Fig. 1), comprising the sequence of the 3′ half of oligonucleotide F and not overlapping oligonucleotide E, was examined for its ability to form complexes with DNA-binding proteins present in Huh7 nuclear extracts (Fig. 3). Included in this analysis was an Sp1 double-stranded oligonucleotide, since oligonucleotide B contained a sequence element which was similar to the Sp1 consensus recognition sequence (9). The observation that ^{32}P-labelled double-stranded oligonucleotides F3′ and Sp1 displayed reciprocal inhibition of both gel retardation complexes with excess unlabelled double-stranded oligonucleotides demonstrated that double-stranded oligonucleotides B, D, E, F, F3′, and Sp1 bound the same or similar DNA-binding proteins. This result is consistent with the major surface antigen promoter containing four binding sites for Sp1 or an Sp1-related transcription factor.

Identification of the Sp1 transcription factor binding sites in the HBV major surface antigen gene promoter. The locations of the Sp1 transcription factor binding sites in the major surface antigen promoter were determined by DNase I footprinting analysis, using purified recombinant Sp1 protein (Fig. 4 and 5). Protection of three regions (sites 1 to 3) of both the long (minus) strand (Fig. 4) and short (plus) strand (Fig. 5) was observed. Sp1 footprint sites 1 and 3 correspond closely to regions B and F3′ characterized by gel retardation analysis. Sp1 footprint site 2 is located at the 5′ end of region D (Fig. 1) and overlaps the 3′ end of region C. An Sp1 footprint mapping within region E was not observed. It is possible that the binding of Sp1 to site 3 precludes the binding of Sp1 to region E.

In an attempt to define further the essential binding sites for the Sp1 transcription factor in the major surface antigen promoter, DNase I footprinting analysis was performed by using purified recombinant Sp1 protein and a variety of mutant major surface antigen promoters (Fig. 1 and 5). Since linker scanning mutation LS−105/−68 did not disrupt the site 1 Sp1 footprint, all of the nucleotide sequences required for binding are located upstream of −105 (coordinate 3053). This result is consistent with Sp1 binding to oligonucleotide B and the location of the Sp1 site 1 footprint (Fig. 1). In contrast to the site 1 footprint, the site 2 footprint was lost as a consequence of the LS−105/−68 mutation. This result indicated that sequences upstream of −67 were important for Sp1 binding to site 2. Since linker scanning mutations LS−76/−69 and LS−61/−49 did not affect the site 2 Sp1 footprint, the essential sequences involved in binding Sp1 at this site are located between −68 and −62 (coordinates 3091 and 3097). This finding indicates that the essential sequence for Sp1 footprinting to site 2 includes the last nucleotide of

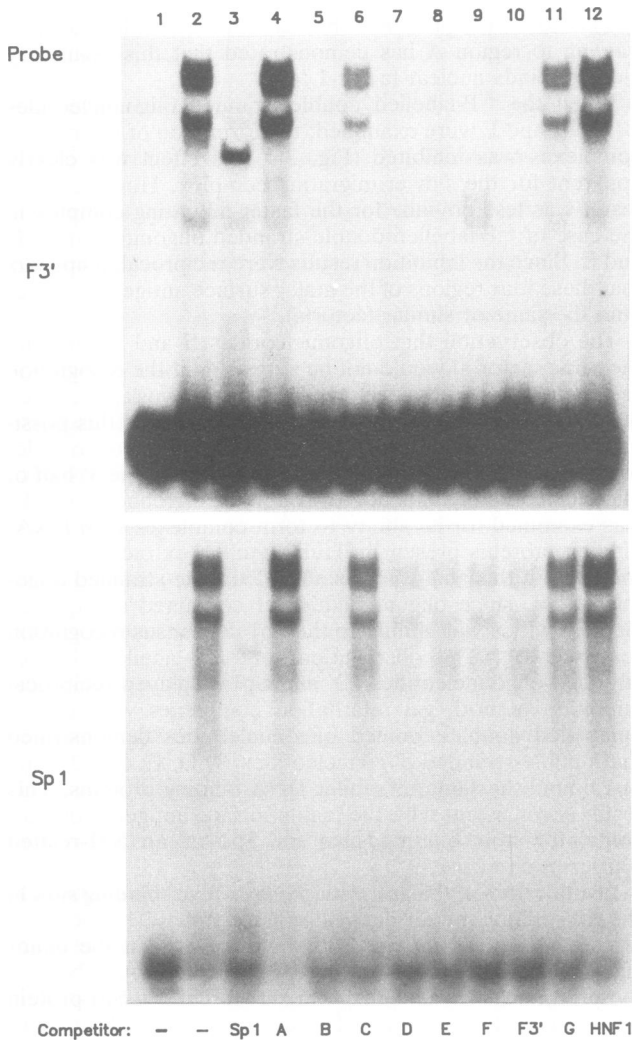

FIG. 3. Identification of Sp1 binding sites in the major surface antigen promoter by gel retardation and complex inhibition analysis. ^{32}P-labelled double-stranded oligonucleotide probes F3′ and Sp1 were analyzed for the ability to form complexes with DNA-binding proteins present in Huh7 cell nuclear extracts. Unlabelled double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. An HNF1 double-stranded oligonucleotide binding site was included as a nonspecific competitor control. Nuclear extract was omitted from lane 1.

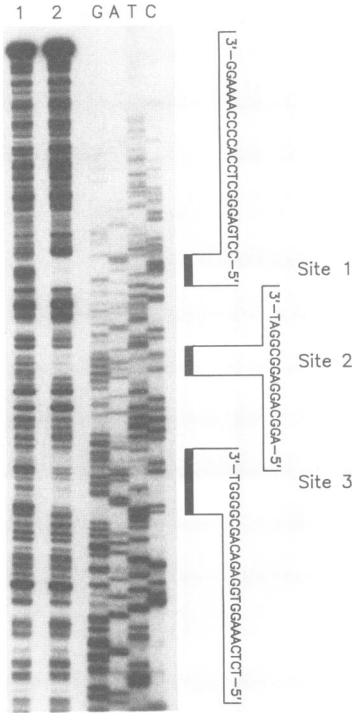

FIG. 4. DNase I footprinting analysis of the long (minus) strand of the HBV major surface antigen promoter. The 476-nucleotide HBV DNA fragment from −319 to +157 (nucleotide coordinates 2840 to 133) was 5′ end labelled at +157 and incubated in the absence (lane 1) or presence (lane 2) of 2.7 U of the Sp1 transcription factor before DNase I digestion. The sequence corresponding to the DNA fragment used for DNase I footprinting is adjacent to lane 2. The sequences of Sp1 sites 1 to 3 are indicated.

region C and the first six nucleotides of region D (Fig. 1). This result is consistent with the location of the site 2 Sp1 footprint identified on the wild-type major surface antigen promoter and probably accounts for the ability of oligonucleotide D to bind Sp1 or an Sp1-related factor in the gel retardation analysis. The observation that linker scanning mutations LS−44/−33, LS−44/−21, and LS−8/+16 do not affect the site 3 Sp1 footprint whereas the LS−22/+16 mutation eliminates this footprint defines the essential recognition sequence of this site between −20 and −9 (coordinates 3139 to 3150) (Fig. 1 and 5). The location of this essential Sp1 recognition element is consistent with the region protected on the wild-type major surface antigen promoter from DNase I digestion by Sp1 and is consistent with oligonucleotide F3′ binding Sp1 or an Sp1-related factor in nuclear extracts. Notably, this analysis failed to generate an Sp1 footprint in region E (Fig. 1). Linker scanning mutation LS−22/+16 prevented Sp1 footprinting of site 3 and did not extend into region E. There are several possible explanations of why this analysis failed to produce an Sp1 footprint in region E. It is possible that the affinity of Sp1 binding to the region E recognition sequence is too low to observe protection in the footprinting assay. Alternatively, an essential recognition element of the region E Sp1 site may be located in region F3′, similar to the observation for the site 2 footprint; i.e., the last nucleotide of region C is essential for observation of the site 2 footprint but appears not to be absolutely required for observation of a gel retardation complex. It appears unlikely that the explanation for the absence of an observed footprint in region E is because region E is recognized by an Sp1-related polypeptide rather than the Sp1 transcription factor (see below).

Sp1 footprinting analysis of the linker scanning mutants (Fig. 1 and 5) also demonstrated that Sp1 can bind to sites 1 and 3 in the absence of binding to site 2 (LS−105/−68) and that Sp1 can bind to sites 1 and 2 in the absence of binding to site 3 (LS−22/+16). This result suggests that the Sp1 transcription factor binds independently to the individual recognition elements in the major surface antigen promoter. This possibility was investigated further by examining the Sp1 footprinting pattern observed in the absence of site 1 (40). Deletion of site 1 did not prevent the observation of Sp1 footprints at sites 2 and 3 (Fig. 1). This evidence supports the

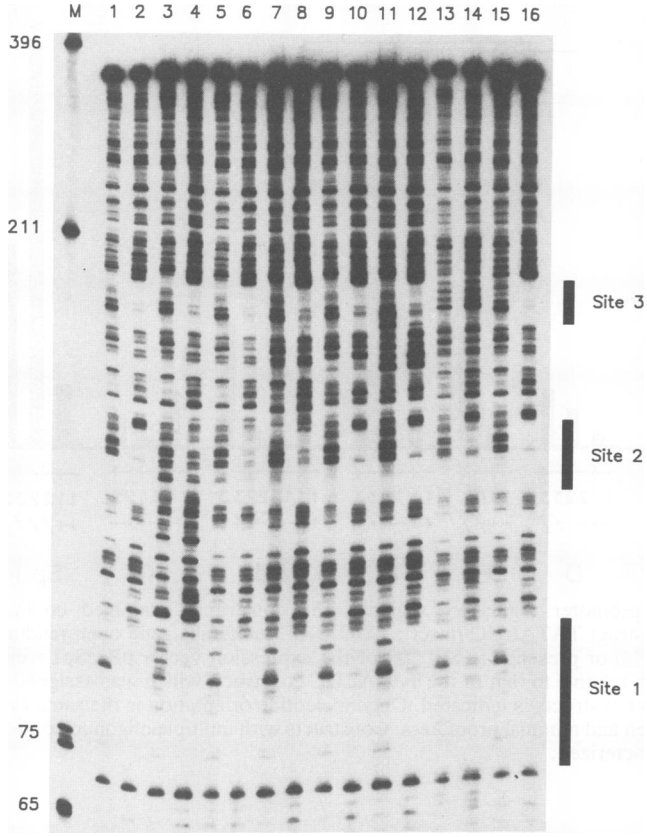

FIG. 5. DNase I footprinting analysis of linker scanning mutants of the HBV major surface antigen promoter. The 345-nucleotide HBV DNA fragment from −188 to +157 (nucleotide coordinates 2971 to 133) was 5′ end labelled at −188 and incubated in the absence (lane 1, 3, 5, 7, 9, 11, 13, and 15) or presence (lanes 2, 4, 6, 8, 10, 12, 14, and 16) of 2.7 U of the Sp1 transcription factor before DNase I digestion. The major surface antigen promoter fragments analyzed were derived from constructs Δ5′−188 (lanes 1 and 2), LS−105/−68 (lanes 3 and 4), LS−76/−69 (lanes 5 and 6), LS−61/−49 (lanes 7 and 8), LS−44/−33 (lanes 9 and 10), LS−44/−21 (lanes 11 and 12), LS−22/+16 (lanes 13 and 14), and LS−8/+16 (lanes 15 and 16). Sp1 sites 1 to 3 are indicated. M, *Hin*fI-digested pUC13 size markers. Sizes are indicated in nucleotides.

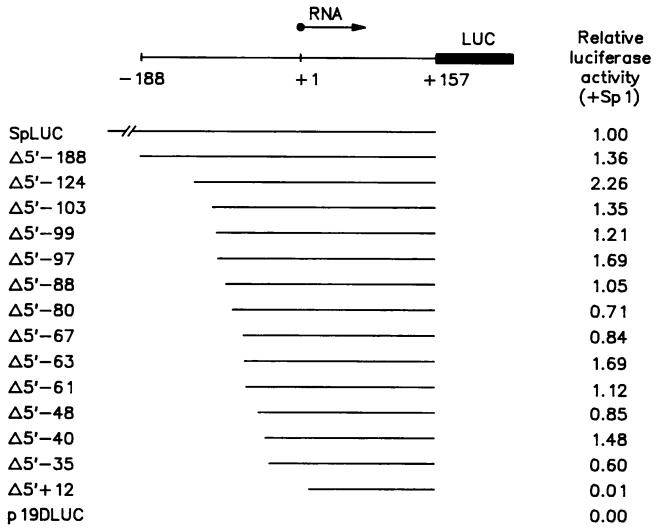

FIG. 6. Deletion analysis from the 5′ end of the HBV major surface antigen promoter. Construct SpLUC contains one complete HBV genome located directly 5′ to the promoterless firefly luciferase (LUC) reporter gene such that expression of this gene is governed by the major surface antigen promoter. Horizontal lines indicate the HBV sequences present in the various luciferase constructs. The diagram above the deletions represents construct Δ5′−188 and indicates the 188 nucleotides of 5′ untranscribed sequence, the major transcription initiation site (+1), and the 157 nucleotides of transcribed HBV sequence present in this construct. Similarly, designations of the other 5′ deletions indicate the extent of the promoter region present. The relative activity of the major surface antigen promoter in the presence of exogenously expressed Sp1 derived from the expression vector pPacSp1, determined in *Drosophila* SL2 cells, is indicated. Transcriptional activities are reported relative to that of construct SpLUC, which was assigned a value of 1.0. In the absence of exogenously expressed Sp1, no transcription is observed from any of the major surface antigen promoter constructs examined. The internal control used to correct for transfection efficiencies was p5CCAT.

idea that Sp1 binds independently to each of the three Sp1 sites in the major surface antigen promoter.

Regulation of transcription from the HBV major surface antigen gene promoter by the Sp1 transcription factor. Gel retardation and DNase I footprinting analysis has provided evidence for the interaction of the Sp1 transcription factor with regulatory regions of the major surface antigen promoter. However, this finding does not establish that Sp1 can regulate the level of transcription from this promoter. This possibility was investigated by using transient transfection experiments in *Drosophila* SL2 cells. Initially, the four HBV promoter constructs, SpLUC, XpLUC, CpLUC, and PS(1) pLUC, were examined for their activities in the presence or absence of exogenously expressed Sp1. The major surface antigen promoter was the only transcriptionally active promoter, and its activity was completely dependent on exogenously expressed Sp1. A 5′-deletion series of the major surface antigen promoter was examined for promoter activity in the presence of exogenously expressed Sp1 to determine the minimal promoter element necessary for Sp1-dependent transcription. Deletion of sequences to −35 (coordinate 3124) did not appear to affect the level of Sp1-inducible transcription from the major surface antigen promoter (Fig. 6). This result indicates that Sp1 sites 1 and 2 are not required for maximal promoter activity in this system.

Deletion analysis of the major surface antigen promoter does not indicate which of the regions, E or F, is involved in mediating the Sp1-induced increase in the level of transcription. In addition, regions B and D might be functional with respect to modulating transcription. This issue was addressed by examining the ability of oligonucleotides A to G to direct Sp1-mediated induction of transcription from a minimal promoter construct, TATALUC, containing only a TATA-box sequence (Fig. 7). This analysis clearly demonstrated the ability of the four major surface antigen promoter regions, B, D (and D5′), E, and F (and F3′), to mediate Sp1 transactivation of transcription (Fig. 1 and 7). None of these oligonucleotides directed levels of transcription from the minimal promoter construct as high as those generated by the Sp1 oligonucleotide(s). This result is consistent with the absence of an absolute match to the Sp1 consensus recognition sequence in these elements (Table 1). In addition, the inclusion of nucleotide −68 in oligonucleotide D5′ generated a construct containing the Sp1 site 2 footprint region which

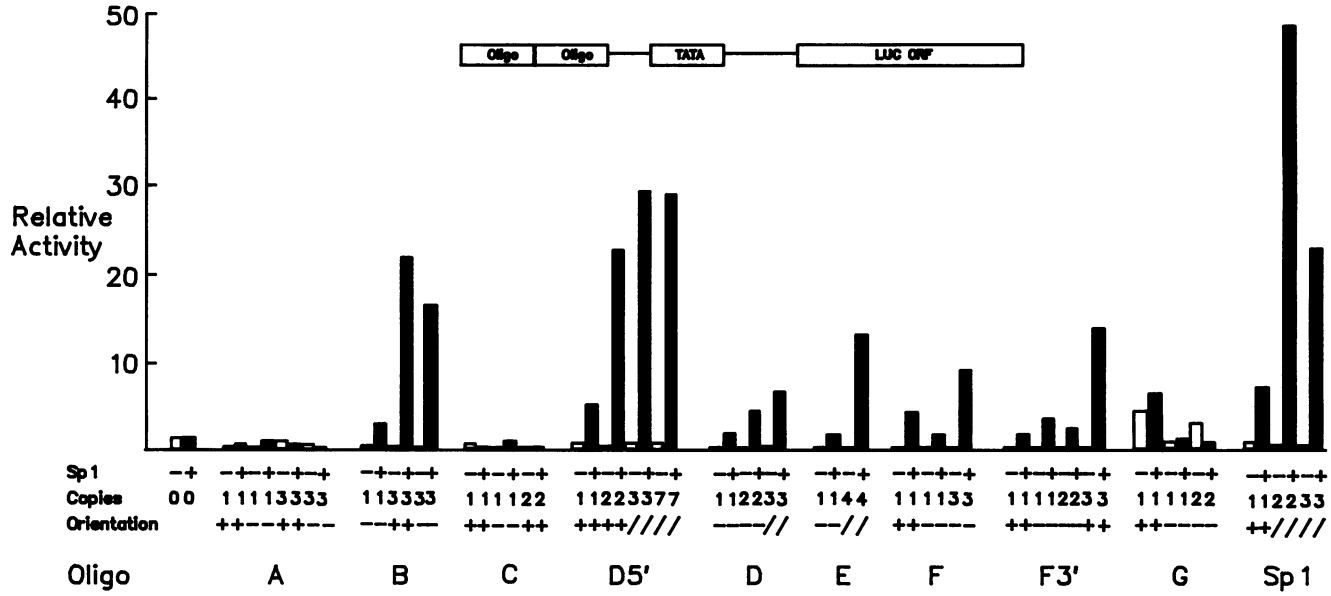

FIG. 7. Functional analysis of the HBV major surface antigen promoter regulatory regions. The constructs examined contain oligonucleotides A to G and Sp1 cloned into the minimal promoter construct TATALUC (insert; LUC ORF, luciferase gene open reading frame). The relative activities of the constructs in the absence (−Sp1; □) or presence (+Sp1; ■) of the expression vector pPacSp1 were examined in *Drosophila* SL2 cells. Transcriptional activities are reported relative to that of the TATALUC construct, which was assigned a value of 1.0. The number of copies of the oligonucleotides in the various constructs is indicated. Oligonucleotide orientation is the same (+) or opposite (−) with respect to transcription from the major surface antigen and minimal promoters. Constructs with multiple oligonucleotides inserted in both orientations (/) in the minimal promoter were also characterized.

was induced to a greater extent than was the oligonucleotide D-containing construct. This result further supports the importance of this nucleotide in the essential recognition element of Sp1 site 2. Oligonucleotides A, C, and G were unable to support Sp1-inducible transcription from the minimal promoter, as would be predicted from results of the gel retardation and DNase I footprinting analysis. However, oligonucleotide G, when oriented in the same direction as it is found in the major surface antigen promoter relative to the direction of transcription, increased transcription from the minimal promoter approximately fivefold, suggesting that this sequence may be interacting with a *Drosophila* transcription factor(s), resulting in the observed increase in transcription. Therefore, these functional studies agree well with the protein binding analysis.

TABLE 1. Comparison between the Sp1 consensus sequence and the HBV major surface antigen promoter Sp1 binding sites

Sp1 site[a]	Sequence[b]	Identity[c]
Sp1 consensus[d]	ATTA TA GCCCCGCCCC	
B region/site 1 (L)	GCTCCaCCCC	9
D5′:D region/site 2 (S)	AaTCCGCCTC	9
E region (S)	AgCCtaCCCC	7
F region/site 3 (S)	tCTCCaCCTt	7

[a] L or S indicates that the sequence is derived from the long or short strand, respectively, of the HBV sequence.
[b] Lowercase letters indicate the nucleotides which differ from the Sp1 consensus sequence.
[c] Number of nucleotides that are identical to the 10-nucleotide Sp1 consensus sequence.
[d] From reference 9.

DISCUSSION

Several lines of evidence indicate that the Sp1 transcription factor has an important role in determining the level of transcription from the HBV major surface antigen promoter. Initially, gel retardation analysis demonstrated that the same or a similar transcription factor(s) bound to the previously identified regulatory regions, B, D, E, and F (and F3′), of the major surface antigen promoter. The observation that the complexes formed with the Sp1 consensus recognition sequence could be inhibited by the B, D, E, and F (and F3′) region oligonucleotides strongly suggested the factor involved was Sp1 or a factor(s) with a related recognition sequence. DNase I footprinting with purified recombinant Sp1 protein demonstrated that Sp1 can bind to regions B, D (or D5′), and F3′ of the major surface antigen promoter. Utilizing transient transfection assays in *Drosophila* SL2 cells, we showed that region B, D, D5′, E, F, and F3′ sequences can mediate Sp1-inducible transcription.

The observation that the Sp1 transcription factor can productively interact with four regions of the major surface antigen promoter was unexpected, as this promoter lacks sequences identical to the consensus Sp1 binding site (9). Inspection of the four regions involved in binding Sp1 reveals the difficulty in identifying the precise nucleotide sequence recognized by Sp1 by using homology to the consensus Sp1 binding sequence (Table 1). The region B Sp1 binding site is identical to the consensus sequence in 9 of 10 positions and probably represents the critical nucleotide sequences involved in binding Sp1. The D5′/D region Sp1 binding site is also identical to the Sp1 consensus sequence in 9 of 10 positions (Table 1). However, there are two reasons why this may not be the sequence element recognized by Sp1. First, only the last five nucleotides of the

consensus sequence are present in oligonucleotide D, which may not be sufficient for it to bind Sp1 in gel retardation and transient transfection assays. Second, the Sp1 site 2 footprint (Fig. 1) is located slightly more 3′ to the location of this sequence than might be expected if this represented the Sp1 recognition sequence. The absence of any other homologies to the Sp1 consensus sequence in this region suggests that this may be the recognition sequence or possibly that Sp1 can interact with sequence elements which are quite divergent from the previously identified consensus sequence. In regions E and F3′, the sequence which is probably recognized by Sp1 is identical to the Sp1 consensus sequence at only 7 of the 10 positions, which might explain why these oligonucleotides mediated a lower Sp1-induced transactivation of transcription from the minimal promoter constructs than did oligonucleotides B and D5′ (Fig. 7).

The observation that the HBV major surface antigen promoter possesses four Sp1 sites has implications for the expression of this gene in mammalian cells. The Sp1 transcription factor is present in a wide variety of mammalian cell types (9, 16), which may partially account for the observation that this promoter is transcriptionally active in nonliver as well as liver-derived cell lines (1, 10, 13, 17, 23, 38, 43, 44, 47, 50). Furthermore, detailed mutational analysis of the major surface antigen promoter demonstrated that transcription from this promoter was regulated by seven regulatory elements, A to G (44). All of these elements positively regulated expression from the promoter except for the region F element, which appeared to inhibit the positive effect that region E had on transcription from the promoter. Although it is difficult to determine the precise role that Sp1 might have in the regulation of this promoter in the presence of the additional transcription factors which interact with the major surface antigen promoter, it is apparent that Sp1 can serve as a positive regulator of expression from this promoter in SL2 cells. Therefore, it is probable that it has the same function in mammalian cells. As regions A, B, and C displayed functional redundancy in human hepatoma cells with regard to surface antigen gene expression (44), it is likely that Sp1 is one of the factors involved in mediating this effect through its interaction with region B. The 5′ region of oligonucleotide D involved in binding Sp1 modulates the level of expression from the major surface antigen promoter approximately threefold (44). This moderate effect might also reflect a level of functional redundancy mediated through the multiple Sp1 sites in this promoter. The most interesting observation is the finding that the oligonucleotide F region which negatively regulates the level of transcription from the major surface antigen promoter in human hepatoma cells under certain circumstances binds the Sp1 transcription factor, which can increase the transcriptional activity from this promoter. However, Sp1 binds to sequences in the 3′ half of oligonucleotide F (Fig. 1), whereas the negative regulatory region of oligonucleotide F is located at the 5′ end, which overlaps with oligonucleotide E (44). The binding of a negative regulatory factor to this location might prevent the binding of Sp1 to the 3′ end of region F or E, which could result in reduced transcription from the major surface antigen promoter. Mutations in the negative regulatory element present at the 5′ end of region F might permit Sp1 binding and the subsequently observed increase in transcription (44). This explanation for the role of Sp1 in regulation of the level of transcription from the major surface antigen promoter is consistent with our previous observations of the regulation of this promoter in human hepatoma cells (44).

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