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Characterization of Functional Sp1 Transcription Factor Binding Sites in the Hepatitis B Virus Nucleocapsid Promoter†

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The hepatitis B virus nucleocapsid minimal promoter contains sequence elements which are similar to the Sp1 transcription factor binding site consensus sequence. The interaction of these regulatory elements with Sp1 was examined by DNase I footprinting with purified Sp1 protein and DNase I footprinting and gel retardation analysis with nuclear extracts from human cell lines and was examined functionally with transient transfection assays in human hepatoma and *Drosophila melanogaster* Schneider line-2 cells. DNase I footprinting identified two regions of the nucleocapsid promoter, representing three recognition elements, that bound purified Sp1. Gel retardation analysis with Huh7 nuclear extracts demonstrated that each of the three recognition elements bound the same or similar transcription factor(s) as that recognized by the Sp1 consensus sequence recognition element. The function of the nucleocapsid promoter elements was examined by transient transfection assays in *D. melanogaster* Schneider line-2 cells 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. In addition, the second Sp1 site was shown to be an essential element of the nucleocapsid promoter in human hepatoma cells. This demonstrates that the hepatitis B virus nucleocapsid promoter contains three functional Sp1 binding sites which may contribute to the level of transcription from this promoter during viral infection.

Hepatitis B virus (HBV) replication is primarily restricted to hepatocytes and proceeds by reverse transcription of a pregenomic RNA transcribed from the viral genome (20, 32). The replication cycle is initiated when the HBV enters the hepatocyte and, by steps which are still poorly defined, delivers the 3.2-kbp partially double-stranded DNA genome to the nucleus where it is converted to the covalently closed relaxed circular form (38, 52). This form of genomic DNA probably represents the template from which the four viral RNAs are transcribed by the host's RNA polymerase. The 3.5-, 2.4-, 2.1-, and 0.7-kb transcripts encode the nucleocapsid and polymerase polypeptides, the large surface antigen polypeptide, the middle and major surface antigen polypeptides, and the X-gene polypeptide, respectively (10, 11, 20, 26, 28, 47, 56). In addition, the 3.5-kb pregenomic RNA and the polymerase polypeptide are incorporated into immature core particles in which pregenomic RNA is converted into the partially double-stranded circular viral genomic DNA (4, 23, 34). Therefore, it is apparent that the 3.5-kb pregenomic RNA has multiple roles in the HBV life cycle, which include encoding viral polypeptides and serving as a replication intermediate. For this reason, the regulation of transcription from the nucleocapsid promoter represents an important aspect of the control of the HBV life cycle.

The regulatory sequence elements which control the level of synthesis of the nucleocapsid transcripts have been characterized in a variety of cell lines (24, 29, 30, 50, 53, 55, 58). Two transcriptional enhancer elements which appear to influence the level of transcription from the nucleocapsid promoter have been identified (1, 27, 31, 35, 45, 51, 58). A minimal nucleocapsid promoter element located within 100 bp of a major 3.5-kb transcription initiation site has also been shown to be important for the activity of this promoter (24, 50, 53, 55, 58). The transcription factors which bind to the enhancers and nucleocapsid promoter sequences and regulate the level of RNA synthesis have not been extensively characterized. One transcription factor, the CCAAT/enhancer binding protein (C/EBP) appears to modulate HBV transcriptional activity from both the enhancer 1 sequence and the nucleocapsid promoter (16, 30, 31, 35, 36, 49). The observation that C/EBP is a liver-enriched transcription factor (5–7, 19) may account, in part, for the highly tissue-specific expression from the nucleocapsid promoter observed during infection and in transgenic mice (2, 18, 38).

Previously, it had been shown by functional analysis and characterization of DNA-protein interactions that the level of transcription from the major surface antigen promoter is controlled, in part, by the Sp1 transcription factor (37). In this study, the role of the Sp1 transcription factor in regulating the level of expression from the nucleocapsid promoter was examined. This analysis demonstrates that there are three functional binding sites for the Sp1 transcription factor located in the regulatory regions of the nucleocapsid promoter (24, 30, 50, 53, 55, 58).

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 (42). The HBV sequences in these constructions were derived from the

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plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the *Eco*RI site of pBR322 (17). The plasmid CpLUC (see Fig. 7) was constructed by digesting pCP10 with *Fsp*I, ligating *Hin*dIII linkers, digesting with *Hin*dIII, and cloning the 3.2-kbp HBV fragment into the *Hin*dIII site of the plasmid p19DLUC (39). The unique HBV *Fsp*I site used in this construct is located 20 nucleotides 3′ of a nucleocapsid gene transcription initiation site (44, 54). Therefore, the plasmid CpLUC contains one complete HBV genome (coordinates 1805 to 3182/1 to 1804) located directly 5′ to the promoterless firefly luciferase (LUC) reporter gene such that the expression of the LUC gene is governed by the HBV nucleocapsid gene promoter. The plasmid constructs containing the various deletions, the CpΔLUC series (see Fig. 7), were generated by *Bal* 31 nuclease digestion or polymerase chain reaction (PCR) synthesis of HBV sequences and subsequent cloning steps similar to those described for CpLUC. All PCR products and deletion breakpoints generated by *Bal* 31 nuclease digestion were confirmed by dideoxynucleotide sequencing (43).

Construction of the TATALUC plasmid (37) was performed by cloning a double-stranded oligonucleotide containing the large surface antigen promoter TATA-box sequence (coordinates 2773 to 2791), produced by annealing the oligonucleotides CTATATTATATAAGAGAGAAGCT and TCTCTCTTATATAATATAGGTAC, into the *Sac*I and *Kpn*I sites of the plasmid p19DLUC (39). Similarly, construction of the CpA, CpB, CpC, CpD, and Sp1 oligonucleotide-containing TATALUC series plasmids (see Fig. 6) was performed by cloning double-stranded oligonucleotides into the unique *Sal*I site of the plasmid TATALUC. The oligonucleotide pairs used to generate the CpA, CpB, CpC, CpD, and Sp1 double-stranded oligonucleotides were TCGACAC CGTGAACGCCCACCAAA and TCGATTTGGTGGGCGT TCACGGTG (oligo-CpA, coordinates 1618 to 1638), TCGA CTGGGAGGAGTT and TCGAAACTCCTCCCAG (oligo-CpB, coordinates 1730 to 1743), TCGAGGGGGAGGAGAT and TCGAATCTCCTCCCCC (oligo-CpC, coordinates 1744 to 1756), TCGATATTGCCCAAG and TCGACTTGGGCAA TA (oligo-CpD, coordinates 1637 to 1648), and TCGAGGGG CGGGGC and TCGAGCCCCGCCCC (oligo-Sp1 [9]). Construction of the Cp-167/-29LUC plasmid was performed by cloning the 139-bp nucleocapsid promoter sequence (coordinates 1618 to 1756) into the *Sma*I site of the plasmid p19DLUC (39) such that expression of the LUC gene is governed by the nucleocapsid promoter. Similarly, construction of the Cp-167/-29TATALUC plasmid was performed by cloning the 139-bp nucleocapsid promoter sequence (coordinates 1618 to 1756) into the *Sma*I site of the plasmid TATALUC (37) in the same orientation with respect to the LUC gene as the Cp-167/-29LUC construct. The coordinates of the nucleocapsid promoter regions are derived from the GenBank genetic sequence data bank.

Cells and transfections. The human hepatoma cell lines Huh7, HepG2, and HepG2.1 (39) were grown in RPMI 1640 medium and 10% fetal bovine serum at 37°C in 5% CO_2-air. The human cervical carcinoma cell line HeLa S3 was grown in Dulbecco's modified Eagle's medium containing 4.5 mg of glucose per ml and 10% fetal calf serum at 37°C in 5% CO_2-air. Transfections were performed as described previously (33, 39, 40). The transfected DNA mixture was composed of 15 μg of a LUC plasmid and 1.5 μg of pSV2CAT (22), which served as an internal control for transfection efficiency. pSV2CAT directs the expression of the chloramphenicol acetyltransferase (CAT) gene by using the simian virus 40 (SV40) early promoter. The *Drosophila melanogaster* Schneider line-2 cells (SL2) 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 (13) with a DNA mixture composed of 1 μg of a LUC plasmid, 200 ng of either the Sp1 expression vector pPacSp1 (13) or the control expression vector pPacU (13), and 10 ng of p5CCAT (8). p5CCAT directs the expression of the CAT gene by using the *D. melanogaster* 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 LUC and CAT activity as previously reported (14, 39, 40).

Nuclear extracts, gel retardation analysis, and DNase I footprinting. Nuclear extracts were prepared from Huh7 and HeLa S3 cells essentially as described previously (15). 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 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 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.

Gel retardation analysis was performed as described previously (36). ^{32}P-labelled double-stranded oligonucleotides (1 ng of each) representing regulatory regions (CpA, CpB, CpC, and CpD) of the nucleocapsid promoter or an Sp1 recognition site (see 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 (9, 40) and contained 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 dithiothreitol, 50 mM KCl, 10% glycerol, and either the purified Sp1 transcription factor (Promega) or 50 μg of the nuclear extract to be assayed. When the nuclear extract was assayed, 1 μg of poly(dI) · poly(dC) was added to the reaction mixture. Binding was carried out for 15 min at 0°C

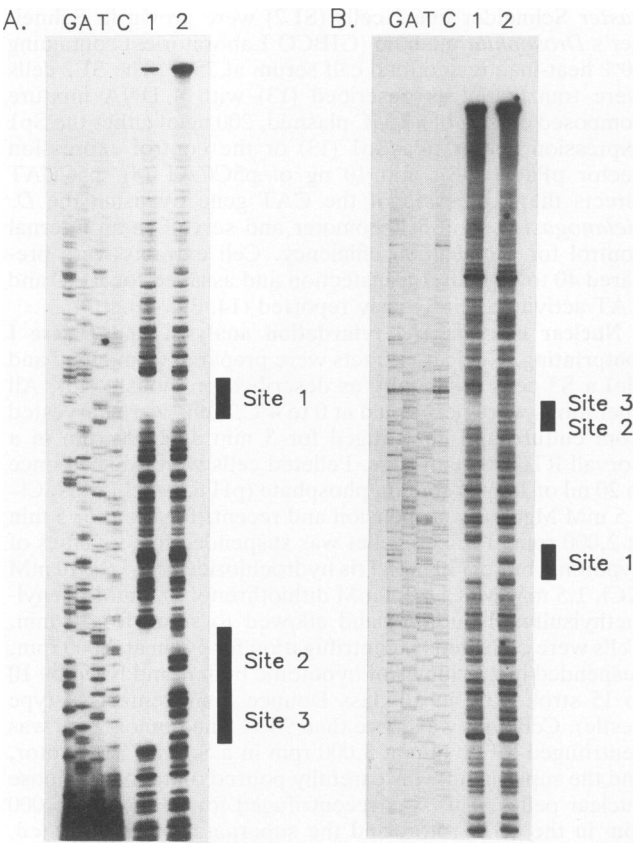


FIG. 1. DNase I footprinting analysis of the long (minus) (A) and short (plus) (B) strands of the HBV nucleocapsid promoter. (A) The 1,119-nucleotide HBV DNA fragment from −1099 to +20 (nucleotide coordinates 686 to 1804) was 5′-end labelled at +20 and incubated in the presence (lane 1) or the absence (lane 2) of 4 U of the Sp1 transcription factor before DNase I digestion. An unrelated DNA sequence (GATC) is adjacent to lane 1 and was used as a size standard to localize the site 1, 2, and 3 footprints (Fig. 8). (B) The 1,808-nucleotide HBV DNA fragment from −410 to +1398 (nucleotide coordinates 1375 to 3182) was 5′-end labelled at −410 and incubated in the presence (lane 1) or the absence (lane 2) of 4 U of the Sp1 transcription factor before DNase I digestion. An unrelated DNA sequence (GATC) is adjacent to lane 1 and was used as a size standard to localize the site 1, 2, and 3 footprints (Fig. 8).

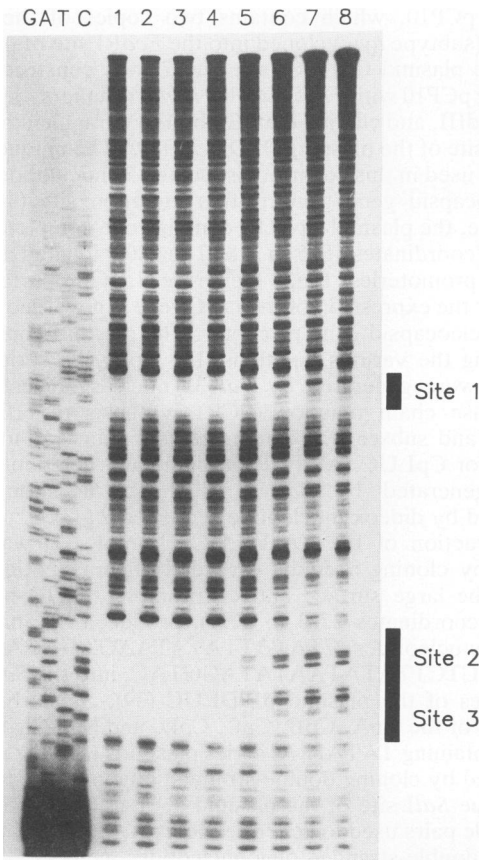


FIG. 2. DNase I footprinting titration analysis of the long (or minus) strand of the HBV nucleocapsid promoter. The 1,119-nucleotide HBV DNA fragment from −1099 to +20 (nucleotide coordinates 686 to 1804) was 5′-end labelled at +20 and incubated in the absence (lane 8) or the presence of 2.7 (lane 1), 1.3 (lane 2), 0.7 (lane 3), 0.3 (lane 4), 0.17 (lane 5), 0.07 (lane 6), and 0.03 (lane 7) units of the Sp1 transcription factor before DNase I digestion. An unrelated DNA sequence (GATC) is adjacent to lane 1 and was used as a size standard to localize the site 1, 2, and 3 footprints (Fig. 8).

followed by 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 reaction mixture was incubated at room temperature for 2 min, and then the reaction was 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 precipitated with ethanol and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

RESULTS

Identification of the Sp1 transcription factor binding sites in the HBV nucleocapsid promoter. The location of the Sp1 transcription factor binding sites in the nucleocapsid promoter was determined by DNase I footprinting analysis with purified recombinant Sp1 protein (Fig. 1 and 2, and see Fig. 8). Protection of two regions (site 1 and sites 2 and 3, respectively) of both the long (L), or minus, strand and short (S), or plus, strand was observed. The Sp1 footprint site 1 spanned nucleotide coordinates 1616 (−169, relative to a predominant transcription initiation site [+1]) (see Fig. 8) to 1638 (−147). The length of the site 1 footprint indicated that a single Sp1 binding site was located at this position of the nucleocapsid promoter (21). Consistent with this observation, it was noted that the central 10 nucleotides of the site 1 footprint showed identity with the Sp1 consensus sequence (9) at seven positions, suggesting that this represented the Sp1 recognition sequence (Table 1).

The second Sp1 footprint spanned nucleotide coordinates 1727 (−58) to 1758 (−27). The length of this footprint indicated that two Sp1 binding sites were located at this position of the nucleocapsid promoter (21). This suggestion was supported by the observation that two 10-nucleotide sequence elements possessing identity with the Sp1 consensus sequence (9) at nine (site 2) and eight (site 3) positions and separated by two nucleotides were located within this DNase I protected footprint region (see Fig. 8; Table 1).

TABLE 1. Comparison between the Sp1 consensus sequence and the HBV nucleocapsid promoter Sp1 binding sites

Sp1 sites[a]	Sequence[b]	Identity[c]
	ATTA TA	
Sp1 consensus[d]	GCCCCGCCCC	
A region, site 1 (S)	tgaACGCCCA	7/10
B region, site 2 (L)	ACTCCtCCCA	9/10
C region, site 3 (L)	tCTCCtCCCC	8/10

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

Therefore, the regions homologous to the Sp1 consensus sequence presumably represent the Sp1 recognition elements in the nucleocapsid promoter.

The three Sp1 recognition elements in the nucleocapsid promoter have different sequences and display differing degrees of homology to the Sp1 consensus sequence (Table 1). The possibility that Sp1 might bind preferentially to a subset of the three nucleocapsid Sp1 sites was examined by analyzing the ability of the three sites to compete for limiting quantities of the Sp1 transcription factor by using the DNase I footprinting assay (Fig. 2). It was observed that both footprints were lost at approximately the same Sp1 concentration, suggesting the three sites have similar affinities for Sp1 binding. The affinity of Sp1 for the nucleocapsid promoter appears to be similar to that observed for Sp1 binding to the SV40 early promoter because 0.3 U of purified Sp1 protein yields complete footprints on approximately 16 fmol of the nucleocapsid promoter fragment (Fig. 2). This conclusion is based on the definition of 1 U of Sp1 protein as the amount of protein required to yield a complete footprint on 35 fmol of an SV40 promoter fragment.

Characterization of the DNA-binding proteins from Huh7 cells which interact with the HBV nucleocapsid promoter Sp1 sites. Initially, DNase I footprinting analysis with Huh7 and HeLa S3 nuclear extracts demonstrated that the major footprints generated on the nucleocapsid promoter were very similar to those observed when purified Sp1 protein was used (Fig. 3). This result is consistent with previously reported footprinting analysis of the nucleocapsid promoter which utilized Huh7, HepG2, and HeLa nuclear extracts (30, 57, 59).

The DNase I footprinting analysis with purified Sp1 protein demonstrated binding to three sites in the nucleocapsid promoter. To examine these interactions further and to determine whether Sp1 is the transcription factor which preferentially binds these sites, the DNA-binding proteins which associate with these sites were examined by gel retardation analysis with Huh7 nuclear extracts. Initially, double-stranded oligonucleotides representing the nucleocapsid promoter Sp1 binding sites CpA, CpB, and CpC, a consensus Sp1 binding site, and a control double-stranded oligonucleotide, CpD (see Fig. 8), were examined for their ability to bind factors present in Huh7 cell nuclear extracts (Fig. 4A). This analysis demonstrated that the three nucleocapsid promoter Sp1 sites and the consensus Sp1 binding site, but not the control double-stranded oligonucleotide CpD, bound specifically to a factor(s) in the nuclear extracts because the corresponding cold double-stranded oligonucleotide inhibited the formation of the gel retardation complexes. In addition, on the basis of their migration properties, it appeared that the gel retardation complexes observed with oligonucleotides CpA, CpB, CpC, and Sp1 might be the same or similar. This is consistent with these oligonucleotides binding Sp1 or an Sp1-related transcription factor.

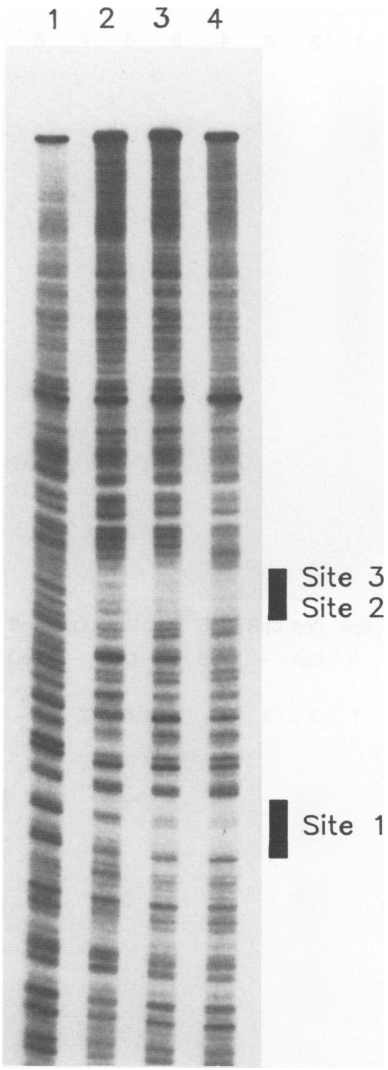


FIG. 3. DNase I footprinting analysis of the short (or plus) strand of the HBV nucleocapsid promoter. The 1,808-nucleotide HBV DNA fragment from −410 to +1398 (nucleotide coordinates 1375 to 3182) was 5′-end labelled at −410 and incubated in the absence (lane 1) or the presence of 50 μg of Huh7 nuclear extract (lane 2), 50 μg of HeLa S3 nuclear extract (lane 3), and 4 U of the Sp1 transcription factor (lane 4) before DNase I digestion. The locations of the Sp1 site 1, 2, and 3 footprints (Fig. 8) are indicated.

The observation that the gel retardation complexes formed with the CpA, CpB, CpC, and Sp1 double-stranded oligonucleotides might be the same was further investigated by examining the inhibition of complex formation by an excess of unlabelled double-stranded oligonucleotides. Initially the inhibition of complex formation with the ^{32}P-labelled double-stranded Sp1 oligonucleotide was examined (Fig. 4B). In this case, the unlabelled double-stranded oligonucleotides CpA, CpB, CpC, Sp1, and D5′ inhibited the formation of both

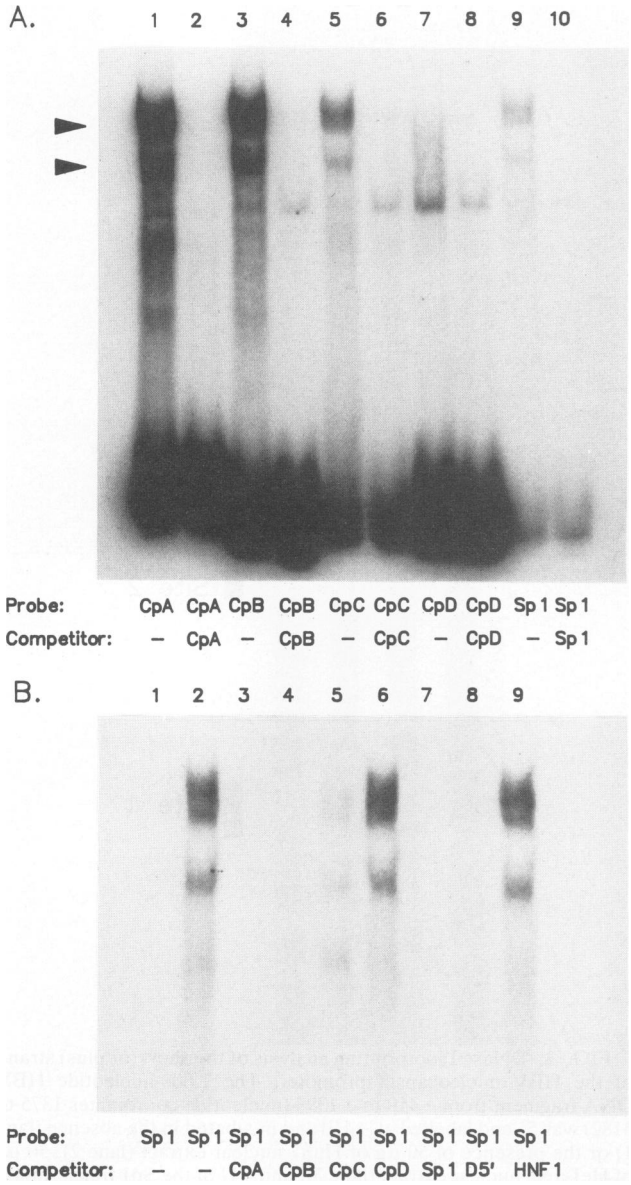


FIG. 4. Gel retardation analysis of the HBV nucleocapsid promoter Sp1 binding regions. (A) The ^{32}P-labelled double-stranded oligonucleotide probes CpA, CpB, CpC, CpD, and Sp1 were analyzed for their ability to form complexes with DNA-binding proteins present in Huh7 cell nuclear extracts. Unlabelled double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. Arrowheads indicate the major complexes which were observed and were sensitive to inhibition by unlabelled double-stranded oligonucleotides. (B) The ^{32}P-labelled double-stranded oligonucleotide Sp1 probe was analyzed for its 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. The D5′ double-stranded oligonucleotide contains an Sp1 binding site present in the major surface antigen promoter (37). A hepatocyte nuclear factor 1 (HNF1) double-stranded oligonucleotide binding site was included as an additional non-specific competitor control (36, 37). Only the complexes which were formed are shown. Nuclear extract was omitted from lane 1.

DNA-protein complexes formed on the ^{32}P-labelled double-stranded Sp1 oligonucleotide, whereas the control unlabelled double-stranded oligonucleotides CpD and HNF1 did not influence complex formation. The D5′ double-stranded oligonucleotide represents an Sp1 binding site from the HBV major surface antigen promoter (37). These results indicate that the CpA, CpB, and CpC double-stranded oligonucleotides can inhibit the formation of the DNA-protein complexes between the double-stranded Sp1 oligonucleotide and Sp1 or Sp1-related transcription factors present in Huh7 cell nuclear extracts.

To investigate further the possibility that the same or similar factors might be binding to the nucleocapsid (CpA, CpB, and CpC) and major surface antigen (B, D, D5′, E, F, and F3′) promoter regulatory sequence elements (37), the effect of an excess of these unlabelled double-stranded oligonucleotides on the formation of the complexes between proteins present in Huh7 cell nuclear extracts and the ^{32}P-labelled double-stranded oligonucleotides CpA, CpB, and CpC was examined (Fig. 5). This analysis demonstrated that the formation of the gel retardation complexes was inhibited when the CpA, CpB, CpC, Sp1, B, D5′, D, E, F, and F3′ double-stranded oligonucleotides were used as competitors (Fig. 5). The CpD and HNF1 (hepatocyte nuclear factor 1) double-stranded oligonucleotides did not affect formation of the DNA-protein complexes. Because the inhibition results were the same for each of these ^{32}P-labelled double-stranded oligonucleotides, it appears that these three regions of the nucleocapsid promoter bind the same or similar transcription factor(s). In addition, the same or similar transcription factor(s) binds four regions of the major surface antigen promoter. On the basis of the DNase I footprinting with purified Sp1 protein and the gel retardation analysis with the Sp1 recognition sequence, it appears that Sp1 or an Sp1-related factor binds to these nucleocapsid promoter regulatory sequence elements.

Regulation of transcription by the HBV nucleocapsid promoter Sp1 binding sites. DNase I footprinting and gel retardation analysis have provided evidence for the interaction of the transcription factor Sp1 with regulatory regions of the nucleocapsid promoter. However, this does not establish that Sp1 can regulate the level of transcription from this promoter. In addition, using transient transfection assays with *Drosophila melanogaster* SL2 cells, the nucleocapsid promoter did not display Sp1-dependent transcriptional activity. In fact the nucleocapsid promoter was inactive in SL2 cells in the presence or absence of exogenously expressed Sp1 (37). Therefore, this analysis did not demonstrate whether the nucleocapsid promoter Sp1 binding sites have the capacity to support Sp1-inducible transcription.

This issue was investigated further with transient transfection experiments in SL2 cells by examining the ability of the CpA, CpB, CpC, and CpD oligonucleotides to direct Sp1-mediated induction of transcription from a minimal promoter construct, TATALUC, containing only a TATA-box sequence (Fig. 6). This analysis clearly demonstrated the ability of the three nucleocapsid promoter regulatory elements CpA, CpB, and CpC to mediate Sp1 transactivation of transcription (Fig. 6). Each of these oligonucleotides directed similar levels of transcription from the minimal promoter construct compared with the Sp1 oligonucleotide when the constructs contained the same number of copies of the oligonucleotide. This indicates that, despite the differences between these recognition sequences (Table 1), these nucleocapsid promoter elements are functionally similar to

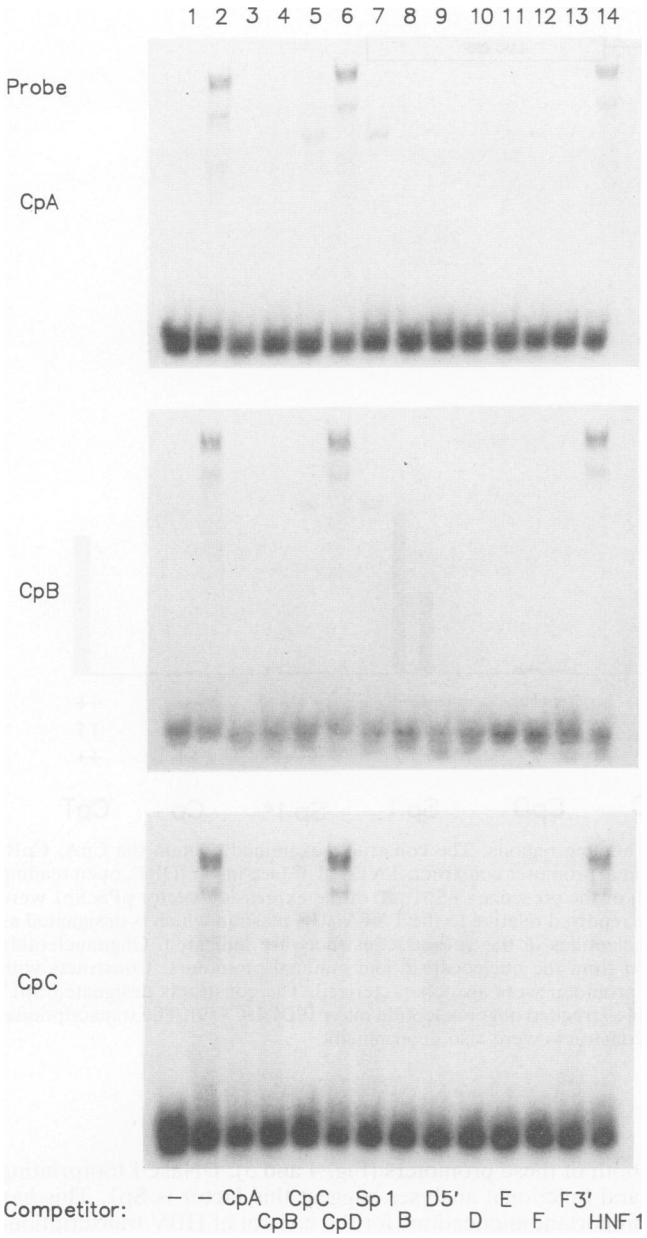


FIG. 5. Gel retardation and complex inhibition analysis of the nucleocapsid promoter Sp1 binding regions. The ^{32}P-labelled double-stranded oligonucleotide probes CpA, CpB, and CpC were analyzed for their ability to form complexes with DNA-binding proteins present in Huh7 cell nuclear extracts. Unlabelled double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. The B, D5′, D, E, F, and F3′ double-stranded oligonucleotides contain Sp1 binding sites present in the major surface antigen promoter (37). A hepatocyte nuclear factor 1 (HNF1) double-stranded oligonucleotide binding site was included as a non-specific competitor control (36, 37). Nuclear extract was omitted from lane 1.

the Sp1 consensus recognition sequence, at least by the criteria being measured in this assay. Oligonucleotide CpD was unable to support Sp1-inducible transcription from the minimal promoter, as would be predicted on the basis of the gel retardation and DNase I footprinting analysis.

Interestingly, when constructs containing the Sp1 consensus sequence but lacking the TATA-box sequence were examined for Sp1-inducible transcriptional activity in SL2 cells, very low levels of transcription were observed only when multiple copies of the Sp1 site were present in the constructs and Sp1 was exogenously expressed (Fig. 6). This observation suggests that the complete nucleocapsid promoter was transcriptionally inactive (37) in transient transfection experiments in SL2 cells because it lacks an appropriately located functional TATA-box recognition element. This possibility is supported by the observation that the construct Cp-167/-29TATALUC, which combines the nucleocapsid promoter sequence from −167 to −29 (coordinates 1618 to 1756, which include the three Sp1 binding sites) with a TATA-box-containing minimal promoter, displays transcriptional activity (Fig. 6). However, in the absence of the TATA-box sequence element, the same nucleocapsid promoter sequence in the construct Cp-167/-29LUC does not display transcriptional activity (Fig. 6).

The transient transfection assays in SL2 cells establish that the nucleocapsid Sp1 sites 1 to 3 can functionally interact with the Sp1 transcription factor to activate transcription. However, the role of the Sp1 sites in the transcription of the 3.5-kb pregenomic RNA in mammalian cells has not been clearly established. Previous analysis in a variety of cell lines demonstrated that the Sp1 site 1 contributed very little to the level of transcription from the nucleocapsid promoter (58). A sequence element located between nucleotide coordinates 1722 (−63) and 1729 (−56) appears to increase transcription from the nucleocapsid promoter approximately fourfold in HepG2 and HepG2.1 cells (compare constructs CpΔ1805-1721LUC and CpΔ1805-1729LUC, Fig. 7). However, the construct CpΔ1805-1729LUC retains approximately 10% of the complete nucleocapsid promoter activity. Deletion of an additional eight nucleotides (compare constructs CpΔ1805-1729LUC and CpΔ1805-1737LUC) spanning the 5′ half of the Sp1 site 2 (Fig. 8) results in the complete loss of promoter activity (Fig. 7). This observation and the binding of Sp1 to this sequence element suggest the importance of the Sp1 transcription factor to nucleocapsid promoter function in mammalian cells.

DISCUSSION

Several lines of evidence indicate that the transcription factor Sp1 interacts with important regulatory regions of the HBV nucleocapsid promoter. Initially, DNase I footprinting with purified recombinant Sp1 protein demonstrated that Sp1 can bind to three sites in the nucleocapsid promoter (Fig. 1 and 8). Double-stranded oligonucleotides containing the individual nucleocapsid promoter Sp1 binding sites identified by DNase I footprinting analysis were subsequently examined by gel retardation analysis. This analysis demonstrated that the same or a similar transcription factor(s) bound to the regulatory sequences present in the CpA, CpB, and CpC regions of the nucleocapsid promoter and the Sp1 consensus recognition sequence. The observation that the Sp1 consensus recognition sequence could also inhibit the formation of these complexes strongly suggested that the factor involved was Sp1 or a factor(s) with a related recognition sequence. Utilizing transient transfection assays of *D. melanogaster* SL2 cells, it was shown that region CpA, CpB, and CpC sequences and a nucleocapsid promoter fragment from −167 to −29 can mediate Sp1-inducible transcription. This demonstrated that the nucleocapsid Sp1 sites were

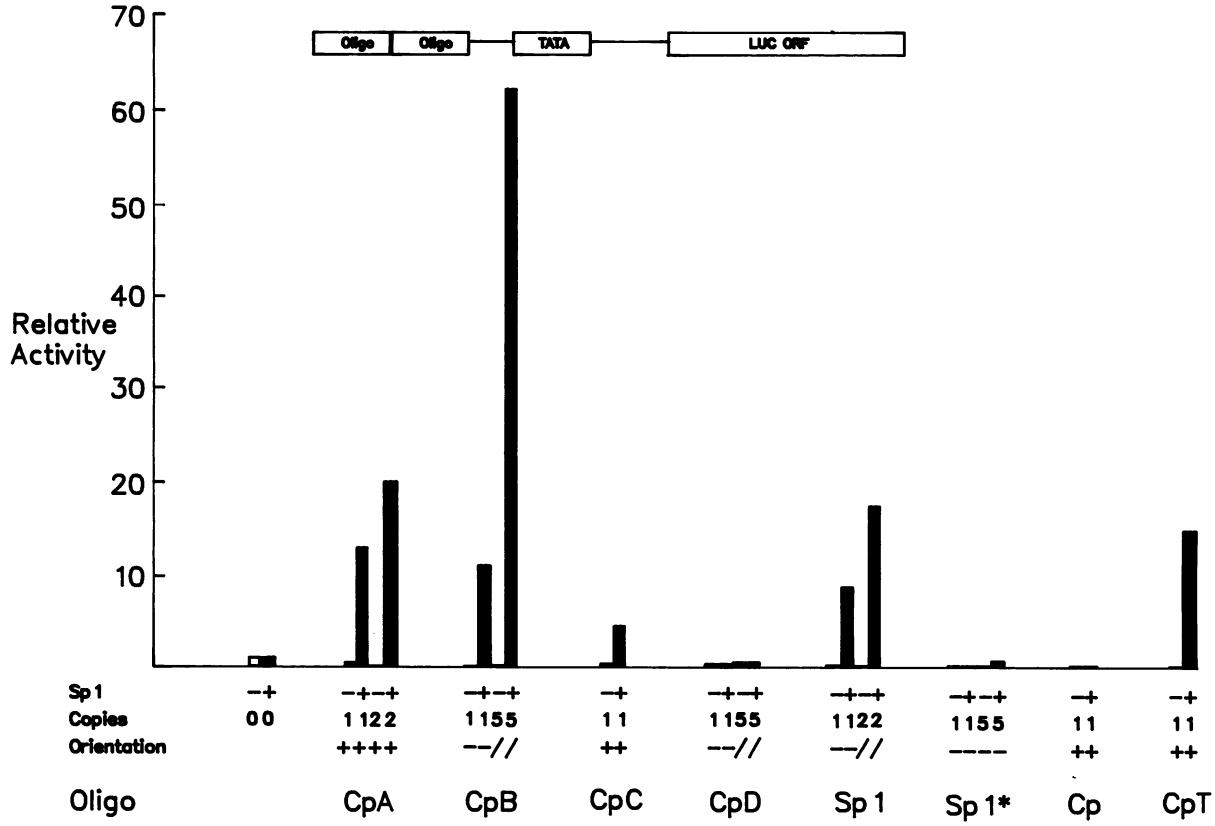
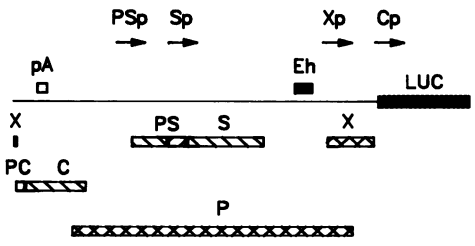


FIG. 6. Functional analysis of the HBV nucleocapsid promoter Sp1 binding regions. The constructs examined contain the CpA, CpB, CpC, CpD, and Sp1 double-stranded oligonucleotides cloned into the minimal promoter construct, TATALUC (see insert [ORF, open reading frame]). The relative activities of the constructs in the absence (−Sp1; □) or the presence (+Sp1; ■) of the expression vector pPacSp1 were examined in *D. melanogaster* SL2 cells. The transcriptional activities are reported relative to the TATALUC plasmid which is designated as having a relative activity of 1.0. The numbers of copies of the oligonucleotides in the various constructs are indicated. Oligonucleotide orientation is the same (+) or opposite (−) with respect to transcription from the nucleocapsid and minimal promoters. Constructs with multiple oligonucleotides inserted in both orientations (/) in the minimal promoter were also characterized. The constructs designated Sp1* lack the TATA-box sequence and were generated by cloning the Sp1 double-stranded oligonucleotide into p19DLUC (39). The transcriptional activities of the Cp-167/-29LUC (Cp) and Cp-167/-29TATALUC (CpT) constructs were also determined.

capable of mediating Sp1-inducible transcription from a minimal promoter despite the failure of the intact nucleocapsid promoter to be activated by exogenously expressed Sp1. Finally, transient transfection assays in human hepatoma cell lines demonstrate that the Sp1 site 2 represents an important sequence element of the nucleocapsid promoter, suggesting that the Sp1 transcription factor may have an important role in determining the level of expression of the 3.5-kb pregenomic RNA in these cells. The observation that the HBV nucleocapsid promoter possesses three Sp1 sites has implications for the expression of this gene in mammalian cells. Since the Sp1 transcription factor is present in a wide variety of mammalian cell types (9, 13), this may partially account for the observation that this promoter is transcriptionally active in non-liver- as well as liver-derived cell lines (24, 29, 30, 50, 53, 55, 58).

This and previous analyses demonstrate that the Sp1 transcription factor appears to have a role in the regulation of the level of transcription from both the nucleocapsid and the major surface antigen promoters (37). From competition gel retardation analysis, it is clear that the same or a similar transcription factor(s) interacts with regulatory regions of both of these promoters (Fig. 4 and 5). DNase I footprinting and functional analyses suggest this factor is Sp1. This has important implications for the control of HBV transcription. In the HBV genome, the nucleocapsid Sp1 binding sites are separated from the major surface antigen Sp1 binding sites by 1.3 kbp. It is known that Sp1 transcription factor molecules bound to different regions of the same DNA molecule and separated by more than 1 kbp can physically and functionally interact to enhance the level of transcription from a promoter (12, 48). However, in our analysis of the regulatory sequence elements controlling the level of transcription from the nucleocapsid and major surface antigen promoters, we have not observed functional modulation of the activity of either promoter by the Sp1 binding site sequences present in the other promoter (37, 40, 41, 58). The absence of any observed functional interaction between these promoters may represent the situation during HBV infection or, alternatively, may represent a consequence of the transfection systems employed to analyze these promoters. Interestingly, functional interactions between sequence elements containing the nucleocapsid promoter Sp1 binding sites and other promoters have been reported. An 88-bp



	Relative luciferase activity	
	HepG2	HepG2.1
CpLUC	1.00	1.00
CpΔ1805–1668LUC	0.27	0.45
CpΔ1805–1721LUC	0.38	0.34
CpΔ1805–1729LUC	0.11	0.09
CpΔ1805–1737LUC	0.00	0.00
CpΔ1805–1743LUC	0.00	0.00
CpΔ1805–1749LUC	0.00	0.00
p19DLUC	0.00	0.00

FIG. 7. Deletion analysis of the HBV nucleocapsid gene promoter. Arrows indicate the positions and directions of transcription from the HBV pre-S1 (PSp), surface antigen (Sp), X gene (Xp), and core (Cp) or nucleocapsid promoters, respectively. Boxes indicate the positions of the HBV enhancer I sequence (Eh), HBV polyadenylation sequence (pA), X gene open reading frame (ORF) (X), presurface antigen ORF (PS), surface antigen ORF (S), precore ORF (PC), core ORF (C), polymerase ORF (P), and luciferase ORF (LUC). The horizontal lines indicate the HBV sequences present in the various CpΔLUC series plasmids. The plasmid CpLUC contains the HBV sequences from nucleotide coordinates 1805 to 3182/1 to 1804 (nucleotide sequences are designated by using coordinates derived from the GenBank genetic sequence data bank). The HBV sequences deleted from the various plasmids are designated by nucleotide coordinates. The internal control used to correct for transfection efficiencies was pSV2CAT.

fragment of the nucleocapsid promoter containing the Sp1 binding sites 2 and 3 was shown to have the properties of a liver-specific enhancer when examined in the context of the herpes simplex virus thymidine kinase promoter (55). Similarly, evidence has been presented that sequence elements containing the nucleocapsid promoter Sp1 binding sites 2 and 3 can modulate the activity of the major surface antigen promoter in a position- and orientation-independent manner

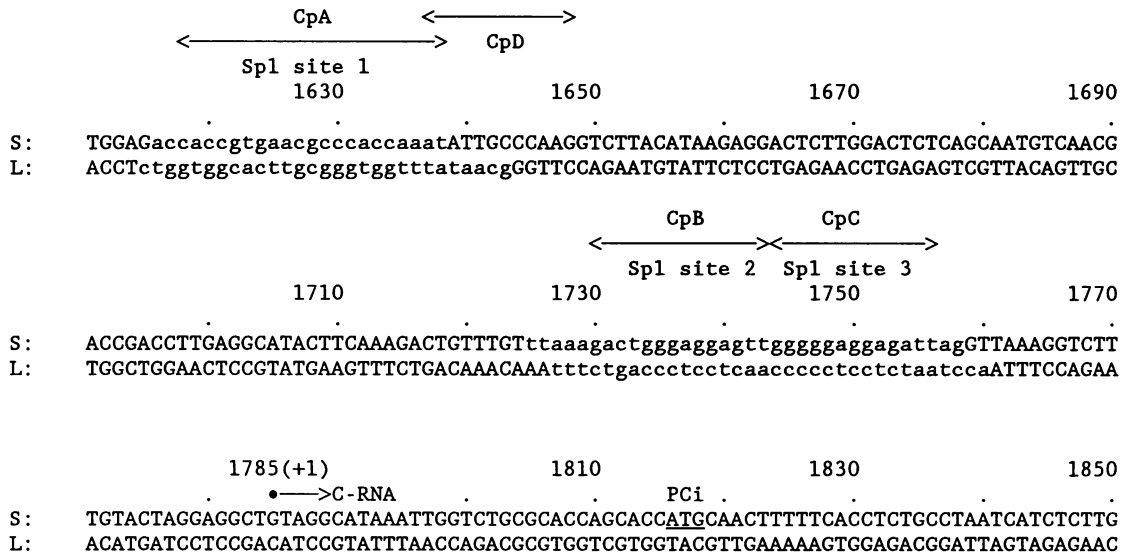


FIG. 8. Sequence of the HBV nucleocapsid promoter region (subtype *ayw*) and the location of the Sp1 footprinting sites 1 to 3. The nucleotide coordinates are shown, and an arrow indicates the approximate location of a predominant HBV nucleocapsid transcription initiation site (+1; nucleotide coordinate 1785) (24, 25, 44, 50, 54). The coordinates of the nucleocapsid promoter region are derived from the GenBank genetic sequence data bank. Lowercase letters represent nucleotides protected from DNase I cleavage by the Sp1 transcription factor. The locations of the Sp1 sites were determined from Fig. 1. The locations of the double-stranded oligonucleotides spanning the Sp1 sites (CpA, CpB, and CpC plus the control CpD) and used in the gel retardation and transfection analyses are also indicated. S, short strand; L, long strand.

(31, 46, 59). However, the specific role of the Sp1 sites in these effects has yet to be established.

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