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Identification of Factor-Binding Sites in the Duck Hepatitis B Virus Enhancer and In Vivo Effects of Enhancer Mutations

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Hepatitis B viruses (hepadnaviruses) can cause chronic, productive infections of hepatocytes. Analyses of the enhancers and promoters of these viruses in cell lines have suggested a requirement of these elements for liver-enriched transcription factors. In this study, a minimum of seven factor-binding sites on the duck hepatitis B virus enhancer were detected by DNase I footprinting using duck liver nuclear extracts. Among the sites that were tentatively identified were one C/EBP-, one HNF1-, and two HNF3-binding sites. Mutations of the HNF1- and HNF3-like sites, which eliminated factor binding, as assessed by both DNase I footprinting and competitive gel shift assays, were evaluated for their effects on enhancer activity. Using a construct in which human growth hormone was expressed from the viral enhancer and core gene promoter, we found that all of the mutations, either alone or in combination, reduced expression two- to fourfold in LMH chicken hepatoma cells. The mutations in the HNF1 site and one of the HNF3 sites, when inserted into the intact viral genome, also suppressed virus RNA synthesis in primary hepatocyte cultures. Virus carrying the latter HNF3 mutation was also examined for its ability to infect and replicate in ducks. No significant inhibition of virus replication was observed in a short-term assay; however, virus with the HNF3 mutation was apparently unable to grow in the pancreas, a second site of duck hepatitis B virus replication in the duck.

Hepatitis B viruses (hepadnaviruses) are highly specific for replication in the liver. Specificity is believed to reflect both the need of the virus to recognize receptors specific to the surface of liver cells and the requirement for the liver's complement of transcription factors for the balanced synthesis of viral mRNAs. A number of studies on transcription from human hepatitis B virus (HBV) DNA fragments transfected into cell lines of diverse origin have in fact supported the idea that transcription of viral DNA to produce at least some of these mRNAs is highly dependent on the presence of transcription factors that are either specific to or enriched in the liver. However, none of these reports indicated whether the transcription factors and HBV transcriptional control elements that have been identified in cell lines are relevant to virus infection and replication in vivo. We therefore undertook a study which addressed both the nature of the transcription factors present in liver extracts and the ability of virus with altered transcription factor-binding sites to replicate in primary hepatocyte cultures or in the liver. These studies were carried out with duck hepatitis B virus (DHBV) and its natural host, the domestic duck.

The template for DHBV RNA synthesis is a unit-length covalently closed circular (CCC) DNA, initially formed from the infecting viral genome and subsequently amplified to ca. 5 to 25 copies per cell by the reverse transcription pathway of viral DNA synthesis (10, 27, 37, 47, 49, 52, 54). Three mRNAs are known to be produced from this CCC DNA in hepatocytes infected with DHBV (5). Studies with transfected cell cultures have also tentatively identified some of the *cis* elements involved in the transcription of these RNAs (5, 13, 35, 45). These include the core, pre-S, and S promoters and a single enhancer, located upstream of the core promoter, that activates transcription from the core and S promoters but not the pre-S promoter. Comparisons of transcription in cell lines of hepatic and nonhepatic origin suggest that transcription from the core and S promoters is liver specific, whereas transcription from the pre-S promoter may have a broader tissue specificity. Moreover, it is clear that part of the restriction of transcription from the core and S promoters in cell cultures is due to their dependence on the viral enhancer and a preference of the viral enhancer for cells of hepatic derivation. In extending these studies to the liver, the normal site of DHBV transcription, three questions have been addressed: What transcription factors in nuclear extracts of duck liver bind to the DHBV enhancer sequence and which of these factors are liver enriched? Are the respective factor binding sites important for enhancer activity in a liver derived cell line? Are the same transcription factors/sites important for virus replication in primary cultures of duck hepatocytes and in ducklings?

In duck liver extracts, at least seven factor-binding sites were apparent within the DHBV enhancer by DNase I footprint analyses. We tentatively identified the factors that bound to several of these sites; thus, the DHBV enhancer apparently contained one C/EBP (28, 29) factor-binding site, two HNF3 (26) binding sites, one HNF1 (11, 12, 39) binding site, and three sites which appeared to bind GATA factors (16, 18). We next examined whether selected factor-binding sites, for HNF1 and HNF3, were important for DHBV enhancer activity. Mutations that destroyed the ability of the individual sites to bind HNF1-like or HNF3-like factors that were present in nuclear extracts caused a two- to fourfold drop in enhancer-mediated expression from the viral core promoter, as assessed in a cell line of liver origin. We then inserted these mutations into the viral genome and assessed the effects on virus replication in cell lines and in primary hepatocyte cultures. None of the mutations had a major effect on virus replication following transfection of a liver cell line. Moreover, the viral particles produced by the mutants seemed to be as infectious as those produced following transfection with the wild type.

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Virus replication was then studied in primary duck hepatocyte cultures. Viruses having a mutation of either the HNF1 site or of one of the HNF3 sites (HNF3/2) replicated almost as efficiently as the wild type, as determined by the intracellular levels of viral RNA and the accumulation in hepatocytes of intermediates in viral DNA synthesis, whereas mutation at the other HNF3 site (HNF3/1) reduced virus replication two- to threefold. In contrast, replication of virus with mutations in the two HNF3 sites, or in both the HNF1 and HNF3 sites, was reduced about 10-fold.

Finally, we examined whether a selected mutant virus, with an HNF3/1 site mutation, was able to replicate in the domestic duck. The mutant virus appeared to replicate in the liver as well as the wild-type virus did, as revealed by viral DNA accumulation in the liver and by levels of virus released into the serum. However, unlike the wild-type virus, which replicates in a subpopulation of cells of the pancreas, virus with a mutation at the HNF3/1 site was unable to replicate in the pancreas.

MATERIALS AND METHODS

Preparation of duck tissue nuclear extracts. Nuclear extracts were prepared from tissues of 14-day-old Pekin ducks (*Anas platyrhynchos domesticus*). To prepare a liver nuclear extract, the liver was perfused with Swimm-77 medium in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM leupeptin as protease inhibitors. The liver was then removed and placed in ice-cold homogenization buffer. All subsequent steps were performed at 4°C. The procedure for making nuclear extracts was modified from that of Tian and Schibler (50). Briefly, the tissue was homogenized, using a tissue homogenizer designed to prevent air intake, in a homogenization buffer containing 2 M sucrose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6), 25 mM KCl, 1 mM EDTA, 10% glycerol, 5% nonfat milk, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM PMSF, 0.5 mM dithiothreitol, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml. The homogenate was centrifuged for 35 min at 24,000 rpm in a Beckman SW27 rotor. The pellet was washed with NE-1 buffer containing 250 mM sucrose, 15 mM Tris-HCl (pH 7.6), 140 mM NaCl, 2 mM $MgCl_2$, and the proteinase inhibitors as described above. The nuclear pellet was then resuspended in NE-2 buffer, which is similar to NE-1 buffer but contains 350 mM KCl, and was then transferred to a Dounce homogenizer. The nuclei were lysed by 20 strokes with pestle A. The viscous lysates were ultracentrifuged for 90 min at 45,000 rpm in an SW60Ti rotor. The supernatant was finally dialyzed with buffer containing 25 mM HEPES (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.1 mM PMSF. The crude extract was aliquoted, quick-frozen in liquid nitrogen, and stored at −80°C. Nuclear extracts from kidney, lung, heart, and spleen were made directly from the organs without perfusion. The protein concentration for the nuclear extracts was about 2 to 3 μg/μl. It should be noted that two measures were found to be critical for making high-quality extracts, one being the use of nonfat milk as a proteinase inhibitor and the other that nuclear proteins are released with 350 mM salt.

Gel mobility shift assay and DNase I footprinting. The gel mobility shift assay was performed as described previously (15). Briefly, 1-ng aliquots of ^{32}P-labeled, double-stranded oligodeoxynucleotides representing different regions of the enhancer region of DHBV (Fig. 1) were incubated at room temperature for 20 min with liver nuclear extract prior to 5% polyacrylamide gel electrophoresis and autoradiography.

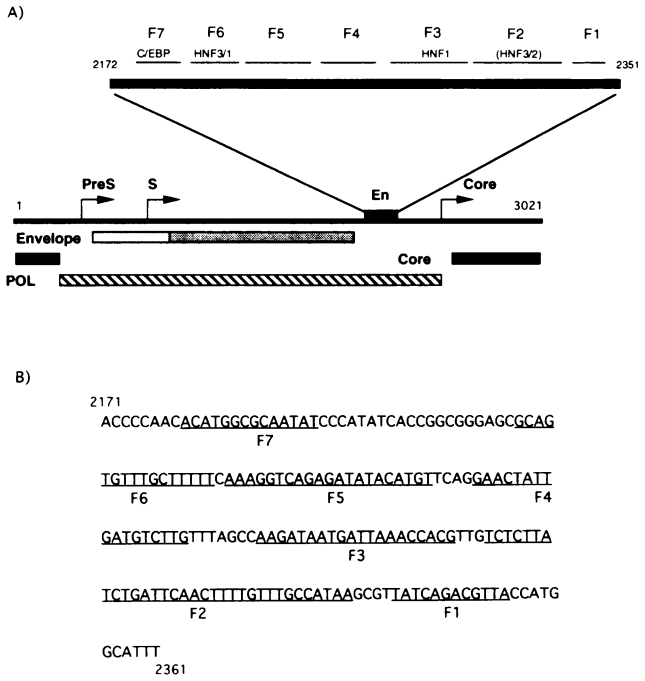

FIG. 1. Locations of transcription factor-binding sites along the DHBV genome. (A) The DHBV enhancer (En), as previously defined (35), is shown along with the locations of DNase I footprints defined in the current study and the apparent identities of transcription factors which contributed, in part, to their generation. (B) The DNA sequence of the DHBV enhancer and of the respective footprints shown in panel A.

When a gel mobility shift competition assay was performed, the nuclear extract was incubated with 100 ng of either the specific or the nonspecific double-stranded competitor oligodeoxynucleotide for 15 min at room temperature. The double-stranded HNF3-specific competitors, TGT3 and TTR, defining HNF3 sites from the mouse albumin gene (24) and mouse transthyretin gene (33), respectively, were kindly provided by Ken Zaret (Brown University, Providence, R.I.). The human HNF1 competitor (defined by a site in the α1-antitrypsin gene) was synthesized according to a published sequence (39), as were the DNAs eG (33) and USF (19). The DHBV-specific double-stranded probes used in the gel shift experiments included HNF1, HNF3/1, HNF3/2, mutant HNF1 (mHNF1), mHNF3/1, and mHNF3/2. The latter three oligonucleotides were also used for mutagenesis of the DHBV enhancer. The sequences of all these DNAs are listed in Table 1.

DNase I footprinting assays were performed as follows. One nanogram of a ^{32}P-end-labeled DNA fragment (DHBV enhancer positions 2172 to 2361) was used as a probe in a total reaction volume of 50 μl. The different nuclear extracts (20 to 30 μg each) were first incubated in reaction buffer containing a final concentration of 12 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM $MgCl_2$, 1% polyvinyl alcohol, 4 μg of poly(dI-dC), 1 mM $CaCl_2$, 5 mM NaCl, 0.1 mM bovine serum albumin (BSA), 0.1 mM dithiothreitol, and 1% glycerol for 15 min on ice; then the probe was added, and incubation continued for another 45 min. The reaction mixture was removed from ice and left at room temperature for 2 min, and the volume was adjusted to 100 μl with reaction buffer. Different amounts of DNase I were added, and the reaction mixtures were incubated

TABLE 1. Sequences of oligonucleotides used in factor binding assays

Oligonucleotide (specificity)	Sequence (5′→3′)
HNF1 (human α1-antitrypsin)	GATCACCTTGGTTAATATTCACCAGCA
TGT3 (HNF3, mouse albumin)	CCGAACGTGTTTGCCTTGGCC
TTR (HNF3, mouse transthyretin)	TGATTCTGATTATTGACTTAGTCA
eG (HNF3, mouse albumin)	CCAGGGAATGTTTGTTCTTAAATACCAT
USF (adenovirus major late promoter)	TAGGTGTAGGCCACGTGACCGGGTGTTCCTG
HNF1 (DHBV, nt 2272–2294)	TTTAGCCAAGATAATGATTAAAC
mHNF1	TTTAGCCAAAATTATGATCAAACC
HNF3/1 (DHBV, nt 2210–2236)	AGCGCAGTGTTTGCTTTTTCAAAGGTC
mHNF3/1	AGCGCAGTATTCGCCTTCTCAAAG
HNF3/2 (DHBV, nt 2312–2338)	GATTCAACTTTTGTTTGCCATAAGCGT
mHNF3/2	GATTCAACCTTCGTCTGTCATAAG

for 2 min at room temperature. Stopping buffer containing 0.1 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 μg of proteinase K per ml, and 50 μg of salmon sperm DNA per ml was then added. This reaction mixture was incubated for 30 min at 37°C, and nucleic acids were then purified by phenol extraction. DNA was precipitated with ethanol at room temperature. The DNA was collected and resuspended in sequence gel loading buffer containing 95% formamide and loaded onto an 8% polyacrylamide sequencing gel. G+A and A sequence ladders of the same DNA used in the footprinting assay were created by the method of Maxam and Gilbert (38).

Site-directed mutagenesis. The single-site mutations were made by using the BioRad Muta-Gene Phagemid in vitro mutagenesis system as recommended by the manufacturer. Briefly, DHBV DNA fragment *Bam*HI (1658)-*Eco*RI (3021) was cloned between *Bam*HI and *Eco*RI sites in the pBluescriptSK(+) polylinker. The recombinant pBluescript(SK+)-DHBV DNA was used to transform *Escherichia coli* CJ236, and the cultures were grown in LB containing 100 μg of ampicillin per ml. The cultures were then infected with the helper phage M13K07. The single-stranded, circular, minus-strand DNA was extracted from the supernatant. Two hundred nanograms of the single-stranded DNA was then annealed with 10 ng of the minus strand of the mutant phosphorylated oligonucleotide mHNF3/1, mHNF3/2, or mHNF1 (Table 1), and T7 phage DNA polymerase was used to synthesize a full-length plus strand from these primers. Following ligation with bacteriophage T4 ligase, the DNAs were used to transform *E. coli* JM101. The presence of the expected mutations in the cloned DNAs was confirmed by DNA sequencing.

To make human growth hormone (hGH) expression vectors in which hGH transcription is under control of the wild-type or mutant DHBV enhancer (single mutated factor-binding site) and core promoter, a DHBV fragment spanning the enhancer and core promoter was produced by cleavage with *Nco*I (2351) and *Bst*EII (1847), gel purified, and then cloned into plasmid pGH-P6 (35). The mutant DNA fragments were also exchanged with the wild-type *Nco*I-to-*Bst*EII fragment in the DHBV genome of pCMVDHBV-9 vector (55), in which the viral pregenome is expressed under the control of the cytomegalovirus immediate-early promoter.

To insert mutations of both HNF3 sites or the HNF3 and HNF1 sites, the PCR approach was used. Briefly, two flanking oligonucleotides which corresponded to the DHBV sequence from nucleotides (nt) 1745 to 1765 and the hGH/DHBV junction of pGH-P5 (5′-GCCTTGGGATCTCGGCCGCT TAAG-3′, nt 2312 to 2526), respectively, were synthesized (35). The mHNF2 oligonucleotide (see above) and the oligonucleotide spanning the hGH/DHBV junction of pGH-P5 were used to produce PCR fragment I, using plasmid pGH-P5 as the template. The minus-strand mHNF3/2 oligonucleotide and a DHBV sequence-specific nucleotide (5′-AACGCAAT TAGCCACGCTGTC-3′, nt 1745 to 1765) were used to produce PCR fragment II, using as the template pCMVDHBV containing the mHNF3/1 mutation. PCR fragments I and II were then mixed in equal molar ratio and amplified by using as primers the DHBV-specific oligonucleotide (nt 1745 to 1765) and the oligonucleotide spanning the hGH/DHBV junction of pGH-P5. To create a triple mutant combining the HNF1 and HNF3 site mutations, the HNF1 oligonucleotide (see above) and the hGH/DHBV junction oligonucleotide were used to produce PCR fragment I, with pGH-mHNF3/2 as the template. PCR fragment II was produced from the template pCMVDHBVmHNF3/1, using as primers the minus-strand mHNF1 oligonucleotide and the hGH/DHBV junction oligonucleotide. The resulting fragments were digested with *Bst*EII and *Nco*I and cloned into appropriate expression vectors as described above.

Transient transfection of LMH cell cultures. Plasmid DNAs were prepared by the alkaline lysis procedure and further purified by isopycnic centrifugation in cesium chloride (36). Five micrograms of each of the hGH expression plasmid DNAs was cotransfected with 5 μg of pRSV-SEAP (secreted alkaline phosphatase) (4) DNA into LMH cells by the calcium phosphate coprecipitation method essentially as described by Condreay et al. (9). The culture fluids were harvested 72 h after transfection, and hGH and SEAP were assayed as described previously (35). The tissue culture fluids were changed every 24 h. The hGH expression levels presented in results were normalized to SEAP levels in the culture fluids.

Generation of viral particles by transfection of LMH cell cultures and infection of duck hepatocyte cultures. Ten micrograms of each of the pCMV-DHBV DNA constructs described above was transfected into LMH cells. The culture fluids were harvested from 3 days to 9 days posttransfection. The titer of virus particles in the pooled culture fluids was estimated by using the partial pronase digestion procedure designed by R. Lenhoff and J. Summers (University of New Mexico). This procedure is based on the observation that intact virus particles are resistant to pronase digestion, whereas viral DNA is released from viral nucleocapsids by pronase digestion under the same conditions and is therefore susceptible to digestion by added DNase I. Briefly, the culture fluids were clarified by centrifugation at 5,000 rpm for 10 min. To precipitate virus particles, polyethylene glycol was then added to 1 ml of supernatant to a final concentration 10% (wt/vol). The resulting pellet was resuspended in 500 μg of pronase solution per ml and incubated at 37°C for 1 h. Then $MgCl_2$ was added to 20 mM and DNase I was added to 5 μg/ml, and the mixture was

incubated at 37°C for 15 min. The reaction product was then subjected to 1% agarose gel electrophoresis, and standard Southern blot analysis was carried out to detect virion DNA. Virion DNA was quantitated with an AMBIS scanning image analyzer.

The infectivity of virus particles produced by transfected LMH cells was assayed in primary hepatocyte cultures. The primary hepatocyte cultures were prepared from the livers of 2-week-old duckling as described previously (52). Hepatocyte monolayers on 60-mm-diameter tissue culture dishes were infected with approximately 10^7 virions. One day later and at daily intervals thereafter, the culture medium was changed with L15 tissue culture medium. Suramin (100 μg/ml) was included in the medium beginning at 1 day postinfection to block secondary rounds of infection (43). The hepatocyte monolayers were harvested at 8 days postinfection and either stored at −80°C for subsequent extraction of viral nucleic acids or fixed with 75% ethanol–25% acetic acid for immunofluorescence microscopy to detect hepatocytes expressing viral core (nucleocapsid) protein. The preparation of tissue sections from ducklings was done as described by Jilbert et al. (27).

Extraction and analyses of viral DNA and RNA. Total viral DNA and viral CCC DNA were extracted as described by Summers et al. (47) and subjected to Southern blotting following gel electrophoresis in 1.5% agarose essentially as described previously (53). To extract viral RNA, one dish of frozen hepatocytes was thawed at room temperature for 5 min, and then 3 ml of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 7.6), 1.5 mM $MgCl_2$, 2% (wt/vol) SDS, and 200 μg of proteinase K per ml was added. The lysate was collected and homogenized with a Dounce homogenizer and then incubated at 45°C for 1 h. The lysate was next passed through a 21-gauge needle to shear cell DNA. Following adjustment of the NaCl concentration of the lysate to 0.5 M, 30 mg of oligo(dT)-cellulose was added. The mix was rocked gently at room temperature for 30 min. The oligo(dT) with bound RNA was collected by centrifugation and washed twice with 0.5 M NaCl–Tris-HCl (pH 7.6)–10 mM EDTA–0.1% (wt/vol) SDS. The oligo(dT) was then transferred to a 0.4-μm-pore-size Millipore filter unit and washed with low-salt binding solution containing 100 mM NaCl, Tris-HCl (pH 7.6), 10 mM EDTA, and 0.1% SDS. The RNA was eluted with 10 mM Tris-HCl (pH 7.6)–10 mM EDTA–0.1% SDS. The RNA was precipitated by addition of 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate. Viral RNA was detected by Northern (RNA) blot analysis following electrophoresis on a 1.0% agarose gel containing formaldehyde (36). DHBV RNAs were detected by using a genome-length minus-strand riboprobe.

RESULTS

At least seven factors present in duck liver extracts bind to the DHBV enhancer. As a first step in identifying transcription factors that interact with the DHBV enhancer, DNase I footprinting assays were carried out with extracts from a variety of duck tissues, including liver, lung, spleen, heart, and kidney. At least seven footprints, labeled F1 to F7, were identified in a duck liver extract (Fig. 1 and 2). Among these, all seven showed some tissue restriction, though only F6 showed a high degree of tissue specificity (Fig. 3). Sequence comparisons suggested that F3 might correspond to a transcription factor HNF1-binding site (11, 39), that F2 and F6 might correspond to HNF3-binding sites (26), and that F7 might correspond to a C/EBP-binding site (28). The fact that F2 showed a broader tissue distribution than F6 suggested that F2 might be the consequence of more complex factor interactions than F6, despite the suggestion that both could have HNF3-binding activity. The sequence at F1, F2, and F3 suggested the possible presence of GATA factor-binding sites (16, 18) on the DHBV enhancer.

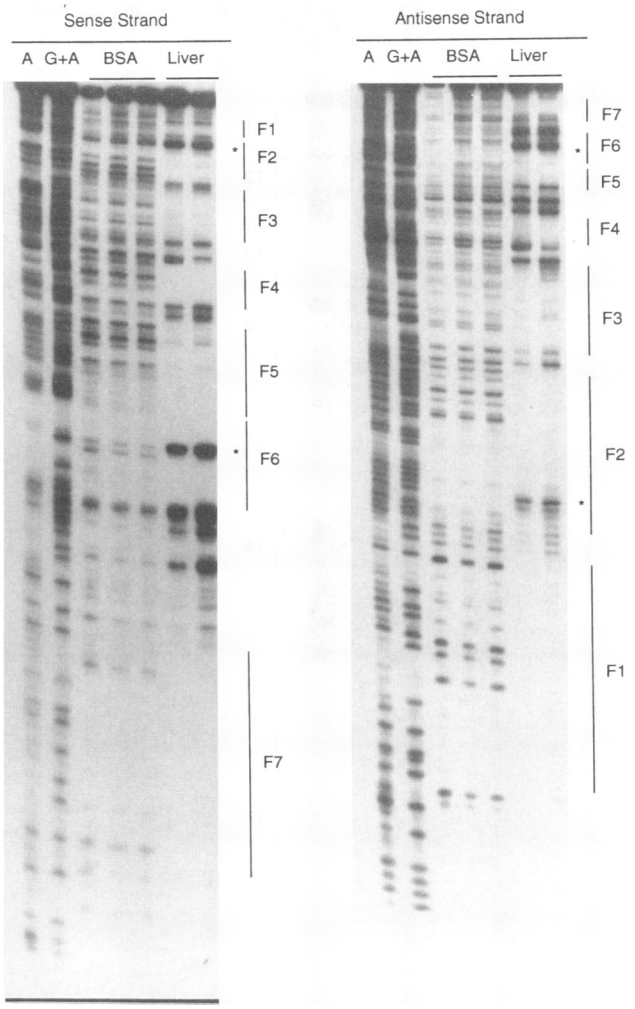

FIG. 2. DNase I footprinting of the DHBV enhancer with duck liver nuclear extract. Locations of the footprints along the DHBV enhancer fragment were determined by reference to A and G+A sequence ladders generated with the same DNA fragment. For the liver extract and BSA controls, the different lanes represent digestions with increasing (from left to right) amounts of DNase I. Hypersensitive sites indicative of HNF3 binding are indicated by asterisks.

Gel shift assays support the identification of potential HNF1 and HNF3 factor-binding sites within the DHBV enhancer. To obtain additional evidence that specific sites identified by DNase I footprinting could have resulted from the binding of transcription factors homologous to those previously identified in other species, competitive gel shift assays were carried out with duck liver extracts. The rationale for the gel shift assays is that binding to a specific DHBV sequence, if due to such a transcription factor, would be blocked by an excess of DNA fragment containing a consensus-binding sequence for that factor but not by consensus-binding sites of other transcription factors. Our analyses focused on HNF1 and HNF3, which appear, from studies with other species, to control some liver-specific transcription during development as well as in the adult liver (57) and which therefore represented

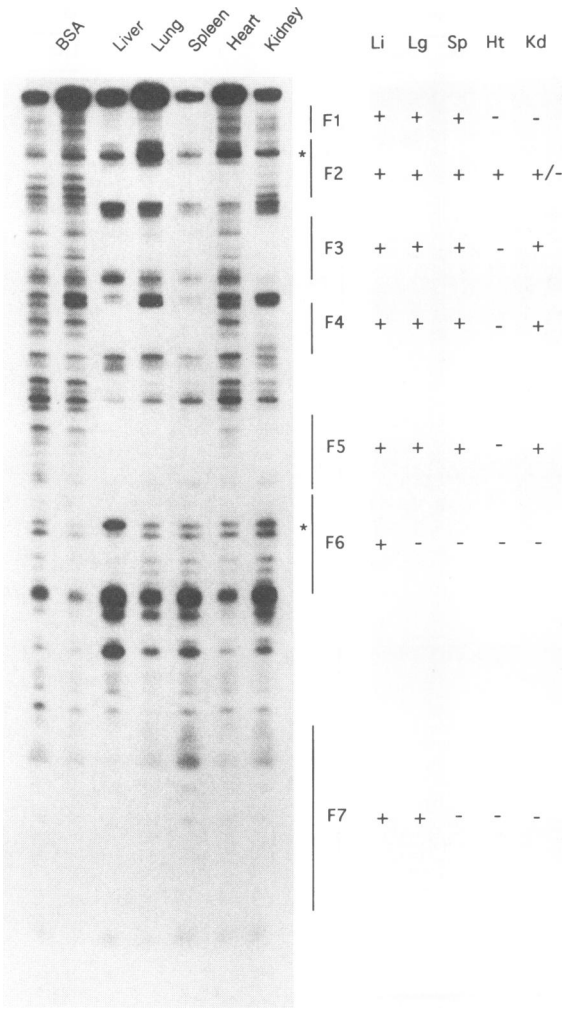

FIG. 3. Tissue specificity of factors binding to the DHBV enhancer. Nuclear extracts were prepared as described in Materials and Methods. Locations of DNase I footprints on the sense strand (see Fig. 2) and their tissue specificity are shown at the right. The tissue assignments of F7 came from data from the other DNA strand (not shown). Li, liver; Lg, lung; Sp, spleen; Ht, heart; Kd, kidney.

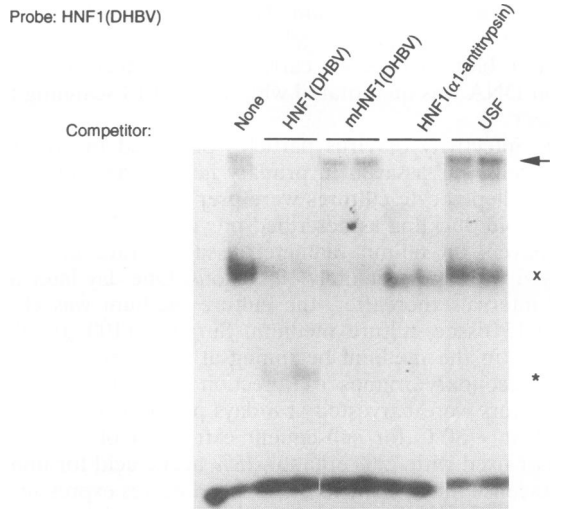

FIG. 4. Competitive gel shift assays indicated that a duck HNF1 binds to the region of DNase I footprint F3. The radiolabeled and unlabeled competitor DNA sequences were as presented in Table 1. The upper band (arrow) is apparently due to interaction with a duck HNF1, as indicated by competition with homologous, unlabeled competitor or with an HNF1-binding site of distinct sequence from the human α1-antitrypsin gene. The middle band may be due to binding by an unrelated transcription factor. HNF1 binding was not competed for by a DNA with a mutant binding site or by the unrelated USF oligonucleotide.

potentially important factors in the facilitation of DHBV transcription.

A competitive gel shift assay using a radiolabeled probe spanning the F3 footprint (Fig. 1), a candidate HNF1-binding site, is shown in Fig. 4. Two prominent bands representing protein-DNA complexes were apparent in the absence of competitor DNA; however, in the presence of competitor homologous to the labeled probe, the upper complex (arrow) was no longer detected and the faster-migrating complex was reduced. We inferred that the upper complex was indicative of an HNF1-like complex, since it was completely eliminated by competition with a DNA containing the well-characterized HNF1-binding site of the human α1-antitrypsin promoter (39) but not by a DNA containing the binding site for transcription factor USF (44) (Fig. 4). The fact that the faster-migrating complex (indicated by x in Fig. 4) was not eliminated by the addition of the α1-antitrypsin sequence suggested that it was due to binding by an unrelated factor. A preliminary experiment (data not shown) indicated that this factor existed exclusively in the liver, but the identity of this factor is presently unknown. The band marked with an asterisk is apparently an artifact of the competition reaction, as a similar band was also observed in other competitions (see Fig. 5 and 7).

The results of competitive gel shift analyses of the two candidate HNF3-binding sites (Fig. 1) is shown in Fig. 5. One major protein-DNA complex was detected (arrow in Fig. 5A) with a probe spanning F6. This complex was reduced by addition of homologous competitor as well as by a competitor spanning F2, the other candidate HNF3-binding site. Effective competition for binding was also obtained with the TTR sequence, representing the high-affinity HNF3-binding site from the transthyretin promoter (33), but competition was less effective with eG or TGT3 DNA, both of which contain lower-affinity HNF3 binding sites from the albumin enhancer (26). With the probe spanning F2, two major complexes were resolved by gel electrophoresis (Fig. 5B). These complexes were effectively competed for with the homologous competitor. However, only one of these complexes (indicated by the arrow in Fig. 5B) was competed for by an excess of a competitor spanning F6 or by eG, TGT3, and TTR competitors, which all contain previously identified HNF3-binding sites. The other complex was not competed for by these DNAs. No competition was observed with use of the USF competitor. In addition, there were some other fast-migrating radiolabeled complexes (Fig. 5B). These complexes were not reproducibly detected; therefore, we believe that these complexes were nonspecific. Taken together with the sequence homologies, these gel shift results suggested that footprints F2 and F6 (Fig. 2) were due in part to binding of a duck transcription factor or factors homologous to HNF3. The site at F6 is referred to as HNF3/1, and the site at F2 is referred to as HNF3/2.

To test the relevance of the factor-binding sites in enhancer

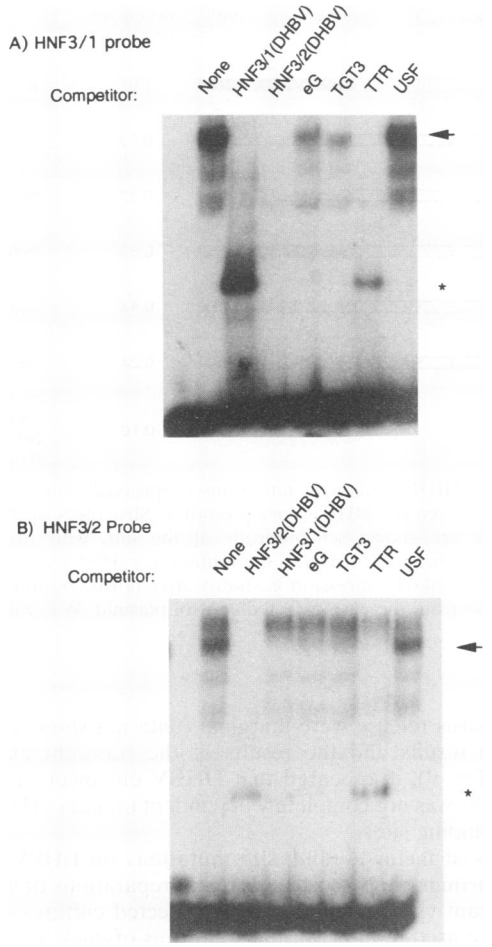

FIG. 5. Competitive gel shift assays indicated that duck HNF3(s) binds to regions F2 and F6 of the DHBV enhancer. The radiolabeled and unlabeled competitor DNA sequences were as presented in Table 1. (A) The HNF3/1 probe from region F6. The arrow indicates the apparent complex with HNF3, as revealed by effective competition with the homologous, unlabeled DNA as well as the HNF3/2 sequence and the TTR DNA containing the HNF3-binding site from the mouse transthyretin gene. Partial competition by the lower-affinity HNF3-binding sequences of eG and TGT3 was also observed. (B) The HNF3/2 probe from region F2. The HNF3 complex, as suggested by the effective competition with DNAs containing heterologous HNF3-binding sites, is indicated by the arrow. The samples in the leftmost lanes in panels A and B contained probe but no liver extract.

function, especially during virus replication, we designed mutants (Fig. 6) that changed the viral DNA sequence without changing the coding capacity of the viral *pol* gene, which overlaps the enhancer. Before inserting these mutations into the viral genome, the respective oligonucleotides (see Materials and Methods) were first tested for the ability to compete in the gel shift assay. As expected, the mutant HNF1 sequence did not block binding of the wild-type sequence in the slowly moving complex (as indicated by arrow in Fig. 4), which appeared to contain an HNF1 transcription factor. Competition was observed, however, with the faster-moving complex, which indicated that the mutant oligonucleotides retained the binding activity to the unknown factor. Our results indicated, therefore, that the mutant DNA bound HNF1 less avidly than the wild-type DNA. Similar results were obtained with the mutations of the HNF3/1- and HNF3/2-binding sites (Fig. 7).

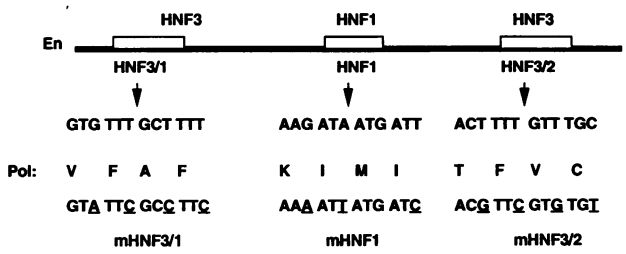

FIG. 6. Third-base mutations introduced into the HNF1- and HNF3-binding sites. Only the mutated region is shown; the sequences of the DNAs used for competitive gel shift assays are presented in Table 1. En, enhancer.

To evaluate further the ability of these mutated binding sites to bind the respective transcription factor, these oligonucleotides were used as probes in gel mobility shift assays. These radiolabeled oligonucleotides were again found to be unable to bind the corresponding transcription factor (data not shown). Finally, when inserted into the viral enhancer, mutations at all

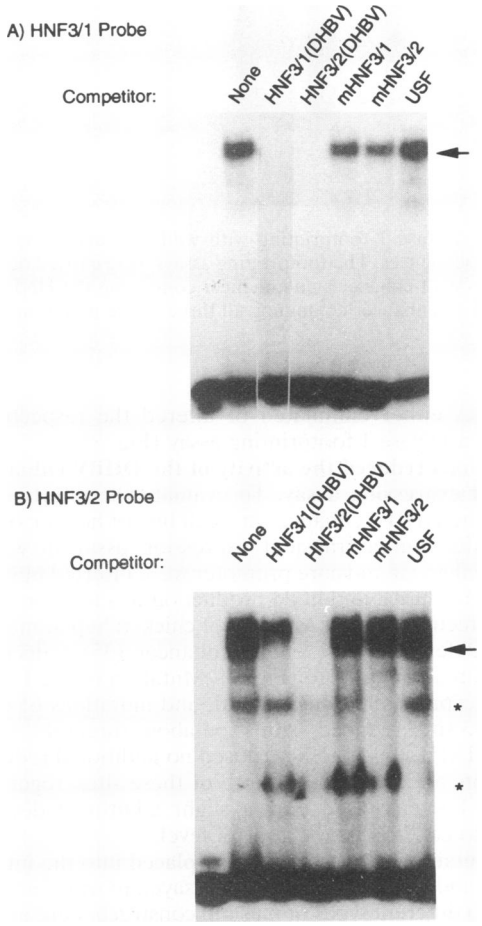

FIG. 7. Mutations at HNF3/1 and HNF3/2 abolished competition for factor binding in gel shift assays. Competition assays using HNF3/1 and HNF3/2 as radiolabeled probes are shown in panels A and B, respectively. Effective cross-competition for duck HNF3 binding (arrow) was observed with the wild-type but not the mutated DNAs. The samples in the leftmost lanes in panels A and B contained probe but no liver extract.

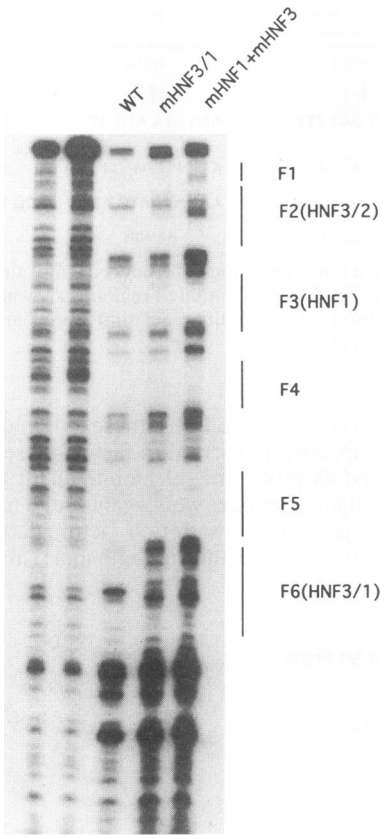

FIG. 8. DNase I footprinting with wild-type and mutant DHBV enhancer sequences. The footprinting assays were carried out with the wild-type (WT) enhancer, an enhancer containing the HNF3/1 mutations, and an enhancer containing all three of the mutations shown in Fig. 6.

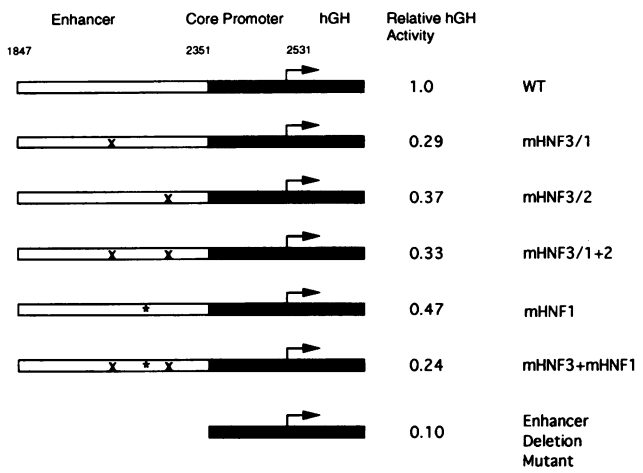

FIG. 9. DHBV enhancer mutations suppressed expression of the hGH gene from the DHBV core promoter: Structures of the various constructs are shown schematically at the left, with the sites of mutations indicated (×, HNF3 site mutation; *, HNF1 site mutation). The level of hGH expression is shown after normalization to SEAP production from the cotransfected control plasmid. WT, wild type.

three sites either eliminated or altered the respective footprints in a DNase I footprinting assay (Fig. 8).

Mutations reduced the activity of the DHBV enhancer in a transient expression assay. To evaluate the effects of mutations in the DHBV enhancer in a cell line of hepatic origin, we first carried out a transient expression assay in which the DHBV enhancer and core promoter were inserted upstream of the gene for hGH, and hGH production was measured following transfection of the LMH line of chicken hepatoma cells (2, 9, 35). Deletion of the entire enhancer (35) reduced hGH production about 10-fold (Fig. 9). Mutation of the HNF1 site reduced expression about twofold, and mutations of either of the HNF3 sites reduced expression about threefold. Mutation of both HNF3 sites together caused no additional reduction in expression, and mutation of both of these sites, together with the HNF1 site, caused only a slight additional decrease in activity, to ca. 25% of the control level.

The mutant enhancers were next placed into the intact viral genome, and virus expression was assayed in transfected LMH cells. Two different types of plasmid constructs were examined. In one, the DHBV genome was cloned at the unique *Eco*RI site (located in the viral core gene; Fig. 1) as a tandem dimer. In the other, DHBV was inserted downstream of the cytomegalovirus immediate-early promoter, which then directed synthesis of viral pregenomic RNA (9). With both types of construct, the effects of the enhancer mutations on the intracellular accumulation of intermediates in viral DNA synthesis and on virus release were marginal (data not shown). In view of these results and the results of the transient expression assays (Fig. 9), it appeared that DHBV enhancer function in LMH cells was not completely dependent on intact HNF1- and HNF3-binding sites.

Effects of factor-binding site mutations on DHBV replication in primary hepatocyte cultures. Preparations of wild-type and mutant viruses released by transfected cultures of LMH cells were used to infect primary cultures of duck hepatocytes. The inocula were adjusted to contain equal amounts of DNA containing viral particles. Suramin was added to the culture medium beginning the day after infection to block secondary rounds of infection (43). The fraction of infected cells was evaluated by immunofluorescent staining of monolayers with serum reactive to DHBV core antigen. A similar number of infected cells was observed by 6 days postinfection with the wild-type virus or with viruses containing multiple mutations of these three factor-binding sites. An approximately threefold-lower percentage of core antigen-positive hepatocytes was detected with the immunofluorescence assay following inoculation of cultures with virus containing mutations in more than one factor-binding site, and those hepatocytes that were core antigen positive had a generally lower signal than seen with the wild-type virus or the single-site mutants. This finding suggests that the immunofluorescence assay may be giving an underestimate of the relative number of hepatocytes infected by the double and triple mutants.

To further evaluate the effects of the different mutations on virus infection, hepatocyte monolayers were extracted, and total and CCC DNA and viral RNA levels were quantitated. As shown in Fig. 10A, mutation at any of the three sites caused, at most, a twofold reduction in total viral DNA accumulation. By contrast, monolayers infected with viruses with mutations at more than one binding site accumulated ca. 10-fold less viral DNA than cells infected with the wild-type virus. Lesser effects on CCC DNA levels were observed, the level of accumulation being reduced only 3- to 4-fold even in infections for which total viral DNA levels were reduced ca. 10-fold (Fig. 10B). The relatively larger amount of CCC DNA could result from

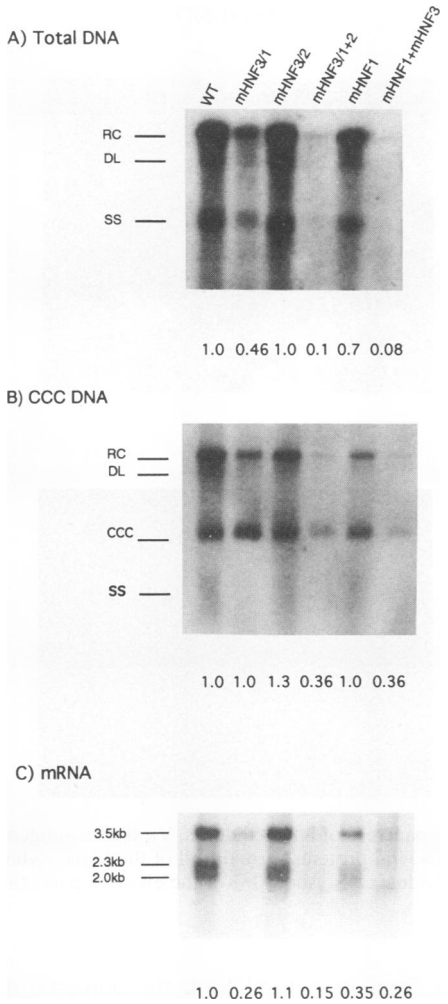

FIG. 10. Effects of mutations in the DHBV enhancer upon virus expression in infected primary duck hepatocyte cultures. Southern blot assays for DHBV DNA in duck hepatocyte cultures at 6 days postinfection are shown in panels A and B, and a Northern blot assay is shown in panel C. (A) Total cell DNA, including DNA replication intermediates; (B) DNA extraction enriched for CCC DNA; (C) viral RNAs in infected hepatocytes. Percent expression of total viral nucleic acids as compared with the wild-type (WT) virus infection is shown below the lanes in each panel. RC and DL, relaxed circular and double-stranded linear 3-kbp viral DNAs, respectively.

selective cycling of newly synthesized viral DNA to the nucleus to elevate CCC DNA levels (47, 48).

The effects of the mutations on viral RNA accumulation are shown in Fig. 10C. As predicted by the transient expression assay in LMH cells (Fig. 9), the effects on viral RNA synthesis in duck hepatocytes were only modest. Mutation of HNF3/2 had no apparent effect on RNA synthesis in the cultures, whereas synthesis was apparently reduced two- to fourfold in cells infected by other mutant viruses.

Effects of enhancer mutations on DHBV replication in ducks. A difficulty in assessing the regulation of viral transcription either in cell lines or in primary cell cultures that have been derived from a particular tissue arises because of the possibility that the regulation may be different in situ. It has been reported that liver-specific transcripts (e.g., albumin mRNA [7, 8]) may be significantly reduced when hepatocytes are maintained in vitro. We have therefore assessed the capacity of one of our mutant viruses, with a mutated HNF3/1 site, to replicate in ducklings. Ducklings at ca. 3 days of age were inoculated intravenously with ca. 10^7 virions per duck. At 2 weeks postinfection, serum samples were collected and assayed for viremia; in addition, the ducks were sacrificed, and the liver and pancreas were monitored for virus replication. DHBV is known to infect and presumably replicate in a small fraction of exocrine cells in the pancreas and probably in the majority of endocrine cells of the pancreas (21, 22, 24, 37). The experimental results are summarized in Table 2. There was no significant difference between the viremias obtained with the wild-type and mutant viruses, though there was some variation between ducklings. To determine the extent of infection in the liver and pancreas, tissue sections were assayed for viral core antigen expression by immunofluorescence microscopy. As illustrated in Fig. 11, core antigen was expressed in the majority of hepatocytes in livers infected by either the wild-type or mutant virus. In contrast, viral core antigen expression was not detected in the pancreas sections of eight of nine of the mutant-infected ducks, though it was detected in pancreas from all six ducklings infected with wild-type virus. Viral DNAs were also isolated from the liver and pancreas tissues of two ducklings infected with wild-type virus and four ducklings infected with the mutant virus and subjected to Southern blot analyses (36a). In agreement with the results of the immunofluorescence assays, we observed similar levels of viral DNA replication intermediates in the livers of wild-type and mutant virus-infected ducks. However, replicative forms of viral DNA were detected only in the pancreas extracts of ducks infected with wild-type virus, not in pancreas extracts from the mutant virus-infected ducks, including the duck that showed core antigen-positive pancreas cells.

To rule out the possibility of contamination with wild-type virus or of reversion of the mutant to wild type as an explanation of the efficient hepatic infections, virion DNA was extracted from the sera of both one wild-type and two mutant virus-infected ducks, and viral enhancer sequences were amplified by PCR. The purified DNA was then sequenced through the region of the original mutation, from 50 bases upstream to 30 bases downstream of the original mutation. No change in sequence as a result of in vivo passage was detected (data not shown).

TABLE 2. Virus with an HNF3/1 mutation can replicate in the duck liver but not the pancreas

Duck no.	Virus	Viremia	DHBV core Ag expression	
			Liver	Pancreas
4	WT	+	+	+
603	WT	+	+	+
604	WT	+	+	+
608	WT	+	+	+
609	WT	+	+	+
1752	WT	+	+	+
7	mHNF3/1	+	+	−
8	mHNF3/1	+	+	−
9	mHNF3/1	+	+	−
10	mHNF3/1	+	+	−
611	mHNF3/1	+	+	+
614	mHNF3/1	+	+	−
616	mHNF3/1	+	+	−
1755	mHNF3/1	+	+	−
1757	mHNF3/1	+	+	−

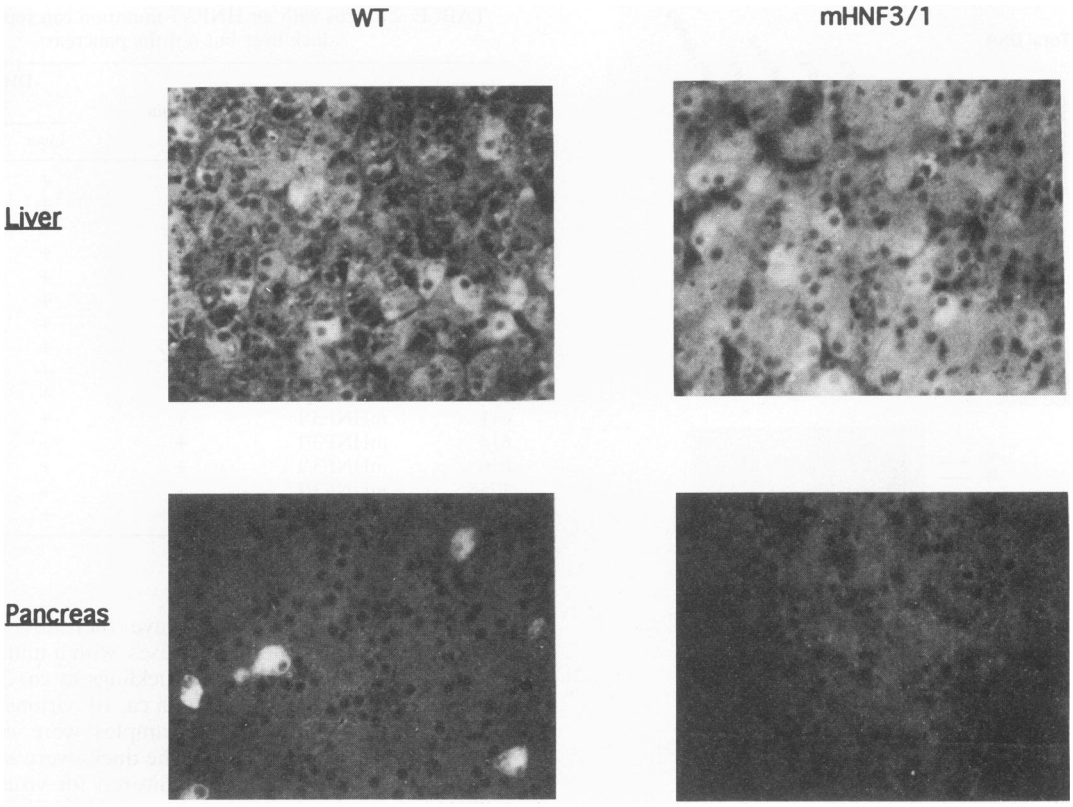

FIG. 11. Virus with a mutant enhancer (mHNF3/1) replicated in the liver but not the pancreas of infected ducks. Viral core antigen expression was detected by immunofluorescence microscopy, using a rabbit antibody reactive to this viral protein. Virtually all of the hepatocytes were core antigen positive, though there was considerable variation in signal intensity between individual cells. About 1% of the exocrine pancreas cells were core antigen positive after infection with the wild-type (WT) but not the mutant virus.

We next carried out a preliminary experiment to determine whether the pancreas was able to support replication of the HNF3/1 mutant virus when the ducks were maintained for longer than 2 weeks. One group of ducks was infected with either wild-type virus or mutant virus. Two ducklings were inoculated intravenously with the mutant virus and two were inoculated with the wild-type virus as described above. By 1 week postinoculation, all four ducks were viremic. The ducklings were sacrificed at 6 weeks postinfection, at which time they were again tested for viremia as well as for infection of the liver and pancreas. With the mutant virus, viremia had fallen to a level that was significantly reduced in the spot test, and though the core antigen was still detectable in the liver, the signal intensity had fallen to very low levels compared with levels found for the wild-type virus-infected ducks. Again, by the immunofluorescence assay, infection of the pancreas was detected only in the two ducks infected by the wild-type virus.

DISCUSSION

Although the transcriptional control elements of HBV have been intensively studied, these studies have depended on the use of either tumor cells of hepatic origin (3, 14, 25, 46, 51, 56) or liver extract from a species such as the rat (42) which is not a host for HBV. To evaluate the role of *cis* elements and host transcription factors in viral transcription, it is important ultimately to carry out studies with liver from the natural host. We therefore chose to study liver-enriched transcription factors affecting the replication of a hepadnavirus by using DHBV and tissues from its natural host, the domestic duck. This required, first of all, optimization of procedures for preparation of nuclear extracts from duck tissues, including but not limited to the liver. Using footprinting analyses and gel shift assays with such extracts, a C/EBP-binding site, an HNF1-binding site, and two HNF3-binding sites were tentatively identified. In DHBV, the sequence (Fig. 1) of the C/EBP site was GCAAT, and that of the HNF1 site was AGATAATGAT TAAAC. The presence of the C/EBP- and HNF1-binding sites in the DHBV enhancer was also recently reported by Lilienbaum et al. (34). The sequence TGTTTGC at both of the HNF3-binding sites (11) which we have described was defined as a new consensus binding site by Jackson et al. (26). This consensus sequence is found in all hepadnaviruses and for some liver-specific host genes (3).

In addition to binding sites for the well-known transcription factors described above, consensus sequences were found in footprints F1, F2, and F3 for the GATA family of transcription factors. The GATA core sequence was initially defined as the binding site for an erythroid cell-specific transcription factor (GATA-1), which has been shown to have an essential role in the activation of a number of erythroid cell-specific genes (16, 17, 40, 41). Several additional GATA factors have now been cloned and characterized. It has been found that these factors have broad spectra of function during development (41). GATA-2 and GATA-3 have high-level expression in mast cells and T lymphocytes (30, 32), respectively. Two new GATA transcription factors (GATA-4 and GATA-5) have recently been cloned from a chicken liver cDNA library (4a). Consid-

ering that the avian liver does not have a hematopoietic function during embryogenesis, it seems likely that GATA factors also have a role in gene expression in hepatocytes. Indeed, mouse (1) and frog (31) homologs of GATA-4 have also recently been identified and shown to be expressed in normal endodermally derived tissues as well as in the heart.

With respect to the number of factors in liver extracts or in other extracts that bind to the DHBV enhancer, it is important to note that our footprinting studies defined a minimum of seven factor-binding sites, and our gel shift assays indicated that there are additional overlapping binding sites that may function with, or in addition to, the sites that we have described. This finding raises the possibility of a level of functional redundancy and may explain why the binding site mutations that we created had such small effects on viral transcription. In making factor-binding site mutations, we had to avoid altering the amino acid sequence of the viral polymerase. Therefore, the only mutations that we made were in the third bases of the respective codons (Fig. 6). This essentially conservative approach to mutagenesis may also have preserved binding by factors other than the ones under investigation. For instance, while it was apparent that as a consequence of mutation, the different sites completely lost the ability to bind HNF1 or HNF3, possible binding of other factors was clearly seen by gel shift analysis.

Because of the presumed importance of liver-enriched HNF1 and HNF3 transcription factors during liver development and hepatocyte differentiation (6, 57), we selected the respective binding sites in DHBV for a study of transcriptional control during virus replication. Though mutations at the HNF1 site and the two HNF3 sites seemed to make the virus enhancer slightly defective in the LMH liver cell line, as expected from the assays with the hGH reporter gene, only one of the three single mutants, with mutations in one of two HNF3-binding sites (HNF3/1), was significantly different from the wild type with regard to its ability to function in primary hepatocyte cultures. Interestingly, the virus with this mutation was able to replicate in duck liver almost as well as the wild-type virus, but it was defective in the ability to infect and/or to replicate in the pancreas. In this regard, it should be noted that the HNF3/1 site was the only site defined in this study for which factor binding appeared to be completely liver specific among the several organ extracts that were tested (Fig. 3); thus, we might have anticipated, a priori, that this site would be especially important for replication in the liver. However, in vivo evaluation of the mutant virus indicated that this was not the case but raised the hypothesis that the site was critical for replication in the pancreas. Unfortunately, we were unsuccessful in several attempts to prepare nuclear extracts from the pancreas with which to test this hypothesis. Therefore, it is equally possible that a factor other than HNF3 may bind to the HNF3/1 site to provide the pancreatic specificity. It has long been known that about 1% of pancreatic cells are able to support DHBV replication. Some of these cells, located in typical acini, have been positively identified as exocrine cells by Halpern and colleagues (21, 23), though the majority of cells located in the acini were not positively identified. In addition to exocrine cells, Halpern and colleagues (20, 24) have also demonstrated that virtually all endocrine cells, located in α and β islets, are targets of DHBV infection. As we were unable to locate endocrine islets in the tissue sections that were available for analysis, we do not know whether the HNF3/1 site mutation also affected virus expression in the endocrine cell compartment. In any case, it would not be surprising if HNF3 were important for DHBV replication in pancreatic cells, inasmuch as liver and pancreas are derived from endoderm, which is influenced by HNF3 during embryogenesis (57). A direct assessment of HNF3 expression in the pancreas would help to resolve this issue, but so far, HNF3 has not been cloned from an avian species. Irrespective of the role of HNF3 in transcription of DHBV, it should be noted that the DHBV results indicate that exocrine pancreas cells, at least those which are targets of infection, have a complement of transcription factors materially different from that of hepatocytes.

One important but unresolved question is whether any single factor-binding site is really important for virus replication in the liver. At least for one of the two HNF3 sites in the DHBV enhancer (HNF3/1), this seemed not to be the case in a short-term assay.

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