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Interplay between a New HNF3 and the HNF1 Transcriptional Factors in the Duck Hepatitis B Virus Enhancer

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We identified a new hepatocyte nuclear factor 3 (HNF3) binding site in the DHBV enhancer. This site is close to the hepatocyte nuclear factor 1 (HNF1) binding site, responsible for most of the enhancing activity. No differences in the migrating properties were found between this new site and the two other HNF3 sites recently described in this enhancer. Factor HNF1 strongly inhibits binding of the HNF3 factor in this newly characterized site. The two factors were never detected simultaneously on the DNA fragment, even when their respective concentrations were modified. Competition persisted after enlarging by 5 and 10 nucleotides the space between the two sites. On the contrary, when the HNF3 binding site was changed into the perfect consensus site, binding of the HNF3 factor was not inhibited any longer by HNF1 and a supershift, corresponding to the binding of both factors, was observed. Thus a limited mismatching appears to modulate the interaction between transcriptional proteins and DNA and allows a second transcriptional protein to interplay with the former one.

INTRODUCTION

The duck hepatitis B virus (DHBV) is a small hepatotropic, not completely double-stranded DNA virus, highly specific for replication in the liver (Mason *et al.*, 1980). Partially explaining this hepatic specificity, a strong enhancer has been localized in the DHBV genome (nt 2159–2351) (Crescenzo-Chaigne *et al.*, 1991; Liu *et al.*, 1991). A further functional dissection has revealed several binding sites for liver-specific transcription factors (Lilienbaum *et al.*, 1993). These liver factors (De Simone and Cortese, 1992) include hepatocyte nuclear factor 1 (HNF1), a member of the POU-Homeobox gene family (Courtois *et al.*, 1988), C/EBP, which belongs to the leucine zipper dimerization family (Landschulz *et al.*, 1988), and HNF3, which shares homology with the Drosophila homeotic gene fork head (Lai *et al.*, 1991, 1993). Together with a fourth factor, HNF4, a member of the thyroid-steroid hormone receptor superfamily (Sladek *et al.*, 1990), these liver-specific transcription factors establish a complex network of interactions and of autoregulation. Thus, HNF4 is known to activate transcription of the HNF1 gene, and HNF1 establishes a negative feedback of its own transcription (Kritis *et al.*, 1993), whereas C/EBP and HNF3 provide a positive feedback of their own transcription. These factors can also interact in a complex manner at the level of the gene they regulate. Among the liver-specific target genes, albumin, transthyretin, α-1 antitrypsin, aldolase B, transferrin, apolipoprotein B, tyrosine aminotransferase, and α-1 microglobulin/bikunin precursor (AMBP) genes bind the same hepatocyte nuclear factors, but each of them are regulated in a specific manner (Jackson *et al.*, 1993; Costa *et al.*, 1989; Gregori *et al.*, 1993, 1994; Petropoulos *et al.*, 1991; Paulweber *et al.*, 1993; Grange *et al.*, 1991; Rouet *et al.*, 1995). The different factor combinations ultimately result in maintaining the right quantitative and specific hepatocyte expression. For instance, on the aldolase B promoter, HNF1 alone has a strong transactivating activity. However, HNF3 behaves as a very strong antiactivator by antagonizing competitively the transcription by HNF1. A second example is given by the AMBP enhancer, where HNF3 has a negative influence over the major HNF4 site as well as a positive influence over the major HNF1 box. Thus specific interactions between hepatic nuclear factors on the enhancers of hepatic genes are responsible for the fine-tuned modulation of their expression.

We report here the identification of a new HNF3 binding site situated in the close vicinity of the formerly recognized HNF1 site. Moreover we characterized a new way of interplay between the factors HNF1 and the new HNF3 on the DHBV enhancer. This HNF3 site, although having a weak affinity for the nuclear factor, accounts for 20% of the whole enhancing activity. HNF1 strongly displaces HNF3 from its binding site in gel shift experiments. This inhibition of the binding of HNF3 does not appear to be due to sterical constraints since insertion of five nucleotides between the HNF1 and the HNF3 DNA binding sites did not modify the gel shift results. At last, when the new HNF3 site was changed into a stronger reference HNF3 binding site (TTR) (Costa *et al.*, 1989), binding of HNF3 was no longer affected by HNF1, and in gel shift experi-

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ments, a new band corresponding to the binding of both HNF1 and HNF3 appeared. These results show that the complex interaction between the liver-specific transcription factors at the protein–protein level also involves the protein–DNA interaction.

MATERIALS AND METHODS

Plasmid constructions and Bal 31 deletions

The plasmid DH23-1 (Lilienbaum *et al.,* 1993) contains, in the MCS (multicloning site) the *Alu*I–*Nco*I 192-bp fragment of the DHBV enhancer (nucleotides 2159 to 2351), upstream of the thymidine kinase (tk) promoter followed by a chloramphenicol acetyltransferase (CAT) gene and the simian virus 40 polyadenylation signals of the PBLCAT2 (Gorman *et al.,* 1982). Deletions from the 5′ or 3′ ends of the enhancer sequence were realized with the Bal 31 exonuclease, and the conserved parts were inserted into the PBLCAT2 vector.

DH23-1 was cut with the *Cla*I (5′ deletions) or *Bam*HI restriction endonuclease (3′ deletions) and incubated for 30 sec to 3 min with 0.1 unit of Bal 31 per μg of DNA in 0.6 *M* NaCl, 12 m*M* CaCl2, 12 m*M* MgCl2, 20 m*M* Tris–HCl, 0.2 m*M* EDTA, pH 8, at 37°. The reaction was stopped by heating for 5 min at 65° and the DNA was precipitated with ethanol. After blunt ending the Bal 31-generated ends with T4 DNA polymerase and the klenow enzymes, the products of deletion were cut again with *Bam*HI (after 5′ deletions) or *Hin*dIII (after 3′ deletions), gel-isolated, and cloned into either *Xba*I blunt/*Bam*HI (5′ deletions) or into *Hin*dIII/*Xba*I blunt PBLCAT2 (3′ deletions). Plasmids were then religated and used to transform *Escherichia coli* strain DH5.

The extent of each deletion was examined by nucleotide sequencing (Sanger method). Sequence reactions were carried out with the sequenase enzyme (Amersham). Ambiguous sequences caused by compression were resolved by using dITP instead of dGTP in the sequence reaction. The sequence reaction was run on a 6% polyacrylamide gel containing 8 *M* urea, 1× TBE (100 m*M* Tris base, 82 m*M* boric acid, 2 m*M* EDTA).

Plasmids were purified with cesium chloride. Two different preparations were tested. The activity of each clone was tested by transfection on HepG2 cells and by CAT assay.

Cell-lines

The differentiated human hepatoblastoma cell-line HepG2 was grown in Dulbecco's Modified Eagle Medium 4,5‰ glucose, supplemented with 10% fetal calf serum (Gibco) and antibiotics.

Transfection and CAT assay

HepG2 cells were plated at 10^6 cells per 60-mm dish and were transfected by the calcium phosphate method (Chen and Okayama, 1987) with 2 to 10 μg of the indicated CAT construction. The cell extracts were prepared 48 hr after transfection. After washing, the cells were resuspended in 150 μl of 0.25 *M* Tris–HCl, pH 7.4, and then subjected for three times to freezing (−10°) and thawing (37°) and cellular debris were pelleted. The transfection efficiency was evaluated by the βgalactosidase activity expressed in cells cotransfected with the RSV βgal plasmid, containing the long terminal repeat of the Rous Sarcoma virus in front of the βgalactosidase gene.

The CAT activity was determined by incubation of 50 μl of the cell extract with 0.1 μCi of ^{14}C chloramphenicol and 4 m*M* acetylCoA at 37° for 1 hr. The reaction was stopped by ethylacetate. The acetylated forms of ^{14}C chloramphenicol were separated by thin layer plate chromatography before counting with a scintillation counter. The CAT activity was calculated by expressing the values of the acetylated forms as a percentage of the total radioactivity associated with the ^{14}C chloramphenicol substrate. Each experiment was repeated at least three times.

Oligonucleotides

Complementary strands of each oligonucleotide in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5, were heated at 85° for 10 min, and annealed by slow cooling to room temperature and used as probes or as cold competitors in gel-mobility shift assays. The oligonucleotides used are described in *Table 1.* Oligonucleotides F4, F5, and F6 contained the HNF3 binding site recognized in the DHBV enhancer. TG3T and TTR represent, respectively, the sequences for the HNF3 factor from the HBV enhancer I (1168–1193) (Ben-Levy *et al.,* 1989; Ori and Shaul, 1995) and from the promoters of the TTR (transthyretin) or α-1 antitrypsin genes (Lai *et al.,* 1990). Oligonucleotides MF4, MF5, and MF6 corresponded to mutated elements of the HNF3 (F4, F5, F6) binding site. F5-F2 (2257–2295) corresponded to the DHBV enhancer containing HNF3 and HNF1 (F2) binding sites (2272–2295). F5-5-F2 corresponded to the HNF3 and HNF1 binding sites with an insertion of 5 nucleotides as a spacer at position 2274. F5-MF2 corresponded to the HNF3 binding site and to the mutated HNF1 binding site (MF2). TTR-F2 contained the HNF3 TTR promoter and the duck HNF1 enhancer site.

Nuclear extracts

HepG2 cells were washed twice with cold phosphate-buffered saline and harvested by scraping in cold PBS. Cells (10^8) were centrifuged at 4000 *g* for 5 min, and the cell pellet was resuspended in 5 ml of 10 m*M* HEPES, pH 8, 50 m*M* NaCl, 0.5 *M* sucrose, 1 m*M* EDTA, pH 8, 0.5 m*M* spermidine, 0.15 m*M* spermine. Nonidet P-40 was added to a final concentration of 0.1% to release the

nuclei. Nuclei were recovered by centrifugation at 3000 *g* for 10 min at 4°. Nuclear proteins were extracted by incubation for 45 min at 4° with gentle rocking in 2 ml of 10 m*M* HEPES, pH 8, 370 m*M* NaCl, 25% glycerol, 0.1 m*M* EDTA, pH 8, 0.5 m*M* spermidine, 0.15 m*M* spermine, in the presence of protease inhibitors (pepstatin, leupeptin, aprotinin, antipain, and chymostatin) at 5 μg/ml each, 1 m*M* phenylmethylsulfonyl fluoride, 2 m*M* benzamidine, and 1 m*M* dithiothreitol (DTT).

Proteins were precipitated with 0.3 g/ml of ammonium sulfate for 16 hr at 4°, centrifugated at 30,000 *g* for 20 min at 4° and dialyzed against 20 m*M* HEPES, 60 m*M* KCl, 1 m*M* DTT, 20% glycerol with the above mentioned protease inhibitors, for 3 hr at 4°. The dialyzate was centrifuged at 15,000 *g* for 10 min, and the supernatant was stored at −80° prior to use. Protein concentration was measured by the method of Bradford (Bio-Rad) with bovine serum albumin as a standard.

Mobility shift assays

Gel mobility shift assays were carried out in a 20-μl reaction volume at room temperature. Each reaction mixture contained 1 μg of poly(dI-dC), 5 μg of crude protein nuclear extracts of HepG2 cells in 5 m*M* Tris–HCl, pH 7.4, 2.5 m*M* EDTA, 0.5 m*M* DTT, 50% glycerol, 25 m*M* NaCl, 2.5 m*M* MgCl2, and 1 ng of the labeled DNA probe. The assembled mixture was incubated for 20 min at room temperature. Then the reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× TBE buffer at 120 V, at room temperature. For competition experiments, various amounts of unlabeled competitor oligonucleotides were added to the reaction. After electrophoresis, the gels were dried and autoradiographed.

In vitro transcription and translation

T7 RNA polymerase were used to synthesize HNF1 or HNF3α mRNA from a *Bam*HI-linearized cDNA template (pGEM-1). *In vitro* translation was performed using nuclease-treated rabbit reticulocyte lysate according to the manufacturer's protocol (Promega), in a final volume of 25 μl. Briefly, mRNA was incubated 1 hr at 30° with 15 μl of rabbit reticulocyte lysate, 1 μl of RNasin ribonuclease inhibitor, 1 m*M* of an amino-acid mixture (minus methionine), and 2 μl of [^{35}S]methionine at 10 mCi/ml.

The translation results were analyzed on a denaturing 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS).

The gel mobility shift assays were carried out under the same conditions with 1 or 0.5 μg poly(dI-dC) and with 1 μl of the crude reticulocyte lysate containing the translated protein.

Mutagenesis

Mutagenesis of the DHBV enhancer was performed by the oligo-directed procedure with the Amersham kit. The DHBV enhancer was cloned into the *Sal*I/*Bam*HI sites of the RF M13 mp18 phage. Single-stranded DNA of the recombinant phage was used as a template for *in vitro* synthesis of the complementary strand. A set of phosphorylated oligonucleotides containing the bases substitution to be inserted were hybridized and then served as primer for the complementary strand synthesis. The sequences of the oligonucleotides are described in Table 1.

The complementary strand was synthesized at 37° by T4 DNA polymerase according to the Amersham's protocol. The mutants were verified by sequencing and the relevant fragments were cloned back into the reporter plasmid PBLCAT2 and transfected into HepG2 cells.

RESULTS

Identification of new sequences in the DHBV *enhancer involved in transcription*

Three binding sites have been previously identified (Lilienbaum *et al.*, 1993): F1 (2180–2200)-C/EBP; F2 (2272–2295)-HNF1, and F3 (2294-2323)-"EFC-like", in the DHBV enhancer. In order to search for the presence of other binding sites not detected by footprinting, Bal 31 deletions were carried out successively at the 3′ or 5′ end of the DHBV enhancer, permitting to obtain clones with deletions of 10 to 30 nucleotides. The DNA fragments were then cloned in a CAT vector (PBLCAT2) and the plasmids were transfected in human hepatoblastoma cells (HepG2). The results obtained are presented in Fig. 1.

The clone 2 (2188–2351) corresponding to a partial deletion of the C/EBP binding site, and clone 3 (2211–2351) to a total deletion of the C/EBP binding site, increased the CAT activity. In contrast, an important fall of activity between clone 3 and clone 4 (2256–2351) was observed after deletion of the F4 fragment. Similarly, there was an important decrease from clone 4 to clone 5 (2276–2351) after deletion of the F5 fragment. Activity was reduced (clone 8) when the nucleotides 2326–2351 (F6) were deleted and when clone 9 (2159–2295) corresponding to the loss of the "F3" site previously described (Lilienbaum *et al.*, 1993) was deleted, the activity still decreased.

Thus new sequences potentially involved in binding of transcription factors appear in the F4, F5, and F6 fragments (Fig. 1). After computer analysis of the DNA sequence, we postulated that the three sequences F4 (2210–2229), F5 (2251–2278), and F6 (2316–2340) could bind the HNF3 protein. F4 and F6 were already described as HNF3 binding sites (Liu *et al.*, 1994) and we used them as reference to analyze the new HNF3 site (F5).

Localization of a new HNF3 site in the close vicinity of the HNF1 binding site

Gel shift assays were carried out to search for binding of the HNF3 protein with the newly identified sequence.

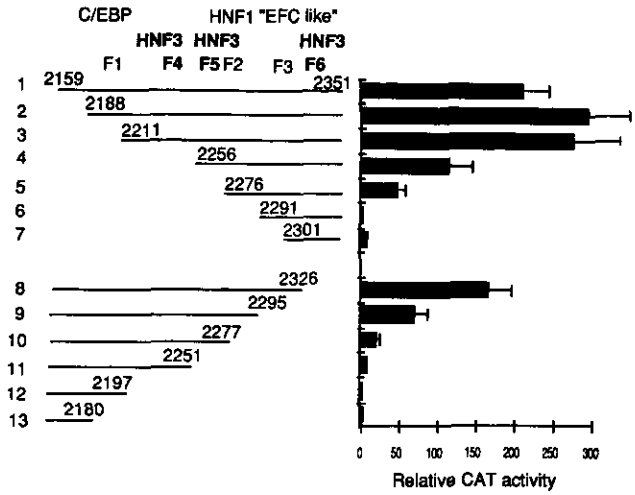

FIG. 1. Enhancing activities of deletion mutants of the duck hepatitis B virus enhancer. The 5′ and 3′ deletion mutants obtained by exonuclease Bal 31 and blunt ended by T4 DNA polymerase and klenow enzymes were inserted in front of the thymidine kinase (tk) promoter and the CAT gene present in the plasmid PBLCAT2. The deletion mutants are represented by lines with nucleotide numbers indicating where they stop (left panel). CAT expression was determined after transfection of the HepG2 cells with every indicated construct (right panel). The efficiency of transfection was controlled by cotransfection with a RSV βgal vector. Transfections were realized with 2 μg of CAT vector, 2 μg of RSV βgal vector, and 6 μg of carrier DNA. The CAT activity of the mutants was evaluated by the βgal activity. The values are the mean values of at least three experiments. The positions of the consensus sequences of the transcription factors F1 to F6 are indicated. The newly identified active sequences are in bold characters.

Oligonucleotides corresponding to the F4 (2210–2229), F5 (2251–2278), and F6 (2316–2340) presumed active sequences were synthesized. The oligonucleotides were labeled, incubated with HepG2 nuclear extracts, and then analyzed in a polyacrylamide gel.

A single DNA–protein complex was detected with the HepG2 nuclear extract for the three probes (Fig. 2, lane 1). The retarded band decreased toward almost complete disappearance when 5- to 100-fold molar excesses of cold competitors were added. (Fig. 2, lanes 2–4). The band also disappeared with oligonucleotides containing two distinct sequences TG3T or TTR, as competitor in the reaction mixture (Fig. 2, lanes 5–7 and 8–10). The nonspecific binding sequence (C/EBP oligonucleotide) used as negative control in the reaction mixture did not compete with the three probes F4, F5, and F6 (Fig. 2, lanes 11–13).

The relative affinity of HNF3 to the three corresponding binding site was analyzed by cross-competition experiments after scanning of the autoradiography. The relative affinity of HNF3 for F4 and F6 was similar. By contrast, the affinity was three times lower for F5 (data not shown).

These results indicate that the F4, F5, and F6 sequences in the DHBV enhancer can specifically bind nuclear factors which are related to the HNF3 family and are displaced by the HNF3 binding sites. To confirm whether the F4, F5, and F6 probes specifically bind the HNF3 protein, gel shift experiments were also carried out using a rat HNF3α protein translated *in vitro* (Lai *et al.*, 1990). A specific complex was detected with the F4, F5, and F6 probes (Fig. 3, lanes 1, 6, 11) and no complex was detected with the negative control probe (Fig. 3, lane 16). The retarded bands significantly decreased as formerly in the presence of the specific unlabeled probe (lanes 2, 3; 7, 8; 12, 13), and incubation of the negative control sequence did not compete with the F4, F5, and F6 probes (lanes 4, 5; 9, 10; 14, 15; 17, 18).

As additional control, the crude reticulocyte lysate without translated proteins, or containing the RNA coding for the luciferase protein or for the rat HNF3 protein incubated with a negative control probe, were tested with the three probes; no significant complexes were detected (not shown). The translated HNF3 protein was analyzed in a SDS–polyacrylamide gel and showed a 46-kDa protein (result not shown) corresponding to the expected size of the rat HNF3 protein.

These results clearly indicate that the sequence F5 coming from the vicinity of HNF1 site to the DHBV enhancer binds the nuclear HNF3 factor exactly like the more distant F4 and F6 sequences do.

Analysis of the transcriptional activity of the HNF3 binding sequences

The enhancing activity was compared to that of the two other HNF3 binding sites to understand how the F5 binding site of the DHBV enhancer contributes to the whole transcriptional activity. Each double-stranded oligonucleotide containing, respectively, the F4, F5, and F6 binding sites was cloned in the sense orientation in a single or in multiple copies in the MCS of PBLCAT2. These clones were used to transfect HepG2 cells, and the amount of CAT activity found in the cell extracts was determined. A very low activity was detected with all constructs, in contrast to that of the complete DHBV enhancer (DH23-1). These results indicate that the HNF3 factor exhibited a poor transactivating efficiency when these new sequences were tested separately (data not shown).

We mutated each HNF3 binding sequence and the HNF1 site in the DH23-1 plasmid to understand the mechanism by which each sequence generates the enhancing activity in the native DHBV enhancer.

After transfection in HepG2 cells, the results of the CAT assay (Fig. 4) show that mutations of the HNF1 site and of the three formerly HNF3 binding sequences induced a decrease of the activity. Mutations in the F4 or F6 fragment resulted in a loss of 60 to 80% of the CAT activity as compared to the native enhancer, and the mutation in F5 (located near HNF1) led to a reduction of 20%. It must be noted that the mutation of the HNF1 site almost completely abolished the transcription (only 10% of the total enhancer activity was remaining unaltered).

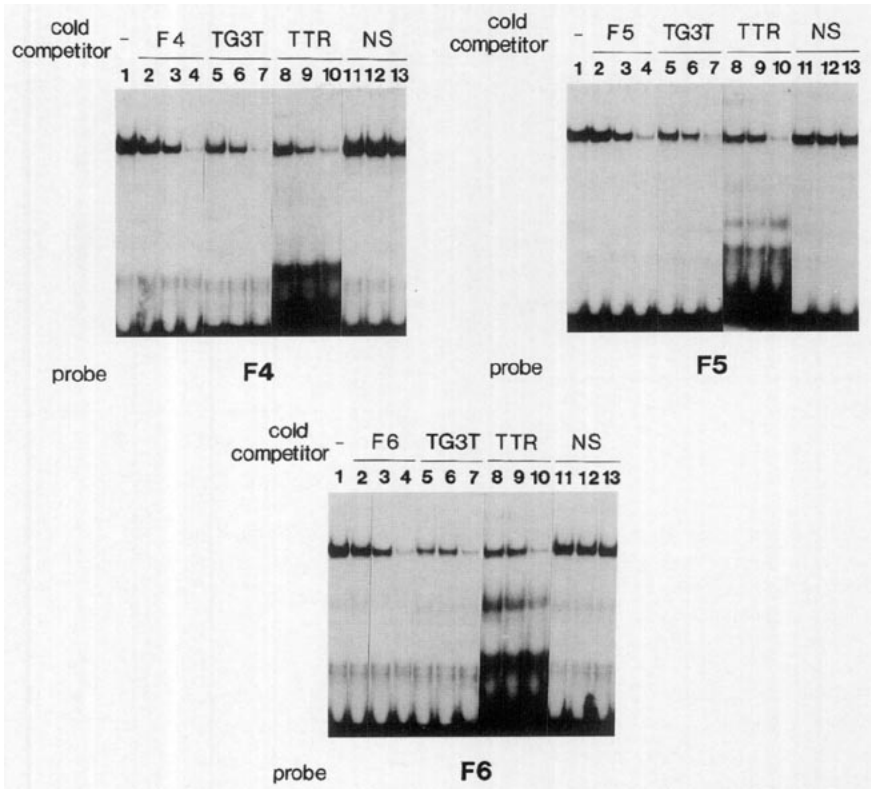

FIG. 2. Binding of nuclear extracts to the presumed active oligonucleotides of regions F4, F5, and F6. HepG2 nuclear extracts (5 μg) were incubated with 1 ng of each ^{32}P-labeled oligonucleotide probe F4, F5, or F6. Increasing amounts of cold competing oligonucleotides were added to the reaction mixtures as follows: lanes 2, 5, 8, and 11, 5-fold molar excess; lanes 3, 6, 9, and 12, 20-fold molar excess; lanes 4, 7, 10, and 13, 100-fold molar excess; lane 1 with proteins and no competitor (−). The assays in lanes 5, 6, and 7 used increasing amounts of cold competitor oligonucleotides encompassing the HNF3 binding sequence from the HBV enhancer I (TG3T); lanes 8, 9, and 10, oligonucleotides with the HNF3 site from the transthyretin promoter (TTR); and lanes 11, 12, and 13 for nonspecific controls (NS: C/EBP oligonucleotides, Bakker *et al.*, 1991). Samples were submitted to electrophoresis in a 5% nondenaturating acrylamide gel; after drying, the gels were autoradiographed.

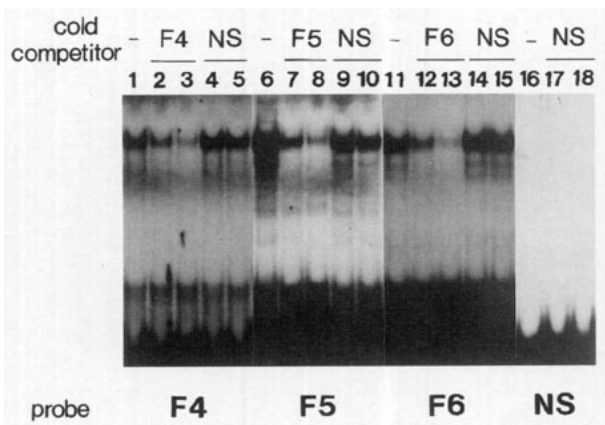

FIG. 3. Binding of the *in vitro* translated HNF3 protein to oligonucleotides encompassing regions F4, F5, and F6. *In vitro* translation of the HNF3 α protein by a mRNA template was incubated with 1 ng of each ^{32}P-labeled oligonucleotide probe F4, F5, F6, or nonspecific control (NS) DNA competitor. Cold competing oligonucleotides were added to the reaction mixtures as follows: lanes 2, 4, 7, 9, 12, 14, and 17, 20-fold molar excess; lanes 3, 5, 8, 10, 13, 15, and 18, 100-fold molar excess; lanes 1, 6, 11, and 16, with proteins and without competitor (−). In lanes 2, 3, 7, 8, 12, 13, 17, and 18, the assays used increasing amounts of cold competitor DNA to the oligonucleotide probe. In lanes 4, 5, 9, 10, 14, and 15, the assays used a NS control DNA competitor.

Loss of the binding capacity of mutated fragments for the HNF3 protein was controlled by the gel shift assay. Competition experiments were performed with the corresponding wild-type oligonucleotides. The three probes F4, F5, and F6 were incubated with the HNF3 protein obtained by *in vitro* translation. Every wild-type oligonucleotide bound the HNF3 protein (Fig. 5, lanes 1, 5, 9). No band could be displaced by an excess of the corresponding cold mutated oligonucleotide (Fig. 5, lanes 3, 4; 7, 8; 11, 12). In contrast, the band disappeared when the corresponding wild-type oligonucleotide was used as competitor (Fig. 5, lanes 2, 6, 10). No complexes were observed when the three mutated oligonucleotides were used as probes (data not shown). These results confirm that the HNF3 protein cannot bind the mutated DNA.

HNF1 inhibits binding of the HNF3 factor in the DHBV enhancer

Given the short distance between the sites of HNF3 (F5) and of HNF1 (F2), a possible competition of both corresponding binding factors was investigated. Therefore an oligonucleotide of 50 nucleotides of DHBV enhancer named F5-F2 containing HNF3 (F5) and HNF1 (F2) binding sites was synthesized and labeled.

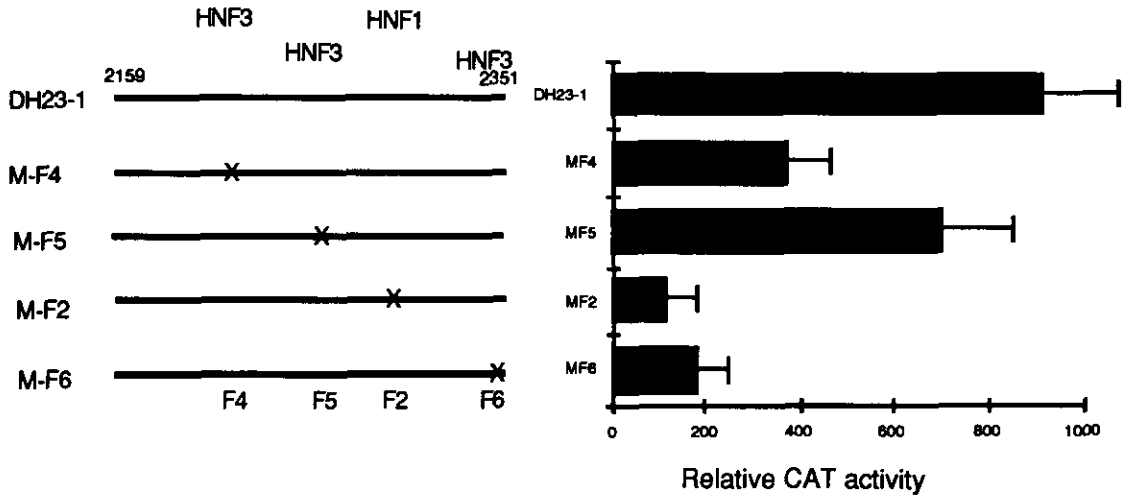

FIG. 4. Enhancing activities of mutants HNF3 binding sequences of the duck hepatitis B virus enhancer. The HNF3 binding sites F4, F5, or F6 were mutated by site-directed mutagenesis. The mutated enhancers were inserted in the PBLCAT2 vector and 10 μg of the plasmid were transfected in HepG2 cells. The left panel shows the position of the mutated sites in the enhancer (crosses). The right panel shows the relative CAT activity calculated from the values of the βgal activities.

At first, binding of the *in vitro* translated HNF3 or HNF1 protein alone to the F5-F2 oligonucleotide was tested by the gel shift assay. Each protein bound the probe individually (Fig. 6A, lanes 1 (HNF3) and 7 (HNF1)). A possibility of competition was then investigated. HNF1 appeared able to block binding of the HNF3 factor to its specifically recognized sequence when similar amounts of both proteins were mixed (Fig. 6A, lanes 4 and 11). Only two complexes were revealed in the presence of the two proteins, and migration of these complexes always corresponded to HNF3 and HNF1 proteins, respectively. As a control, competition experiments with either F2 (HNF1), F5 (HNF3), or F5-F2 (HNF3 + HNF1) oligonucleotides were carried out, and led to disappearance of the band corresponding to HNF1 or HNF3, respectively, or to both proteins (Fig. 6A, lanes 13–15). A hypothetical third complex corresponding to a simultaneous binding of both HNF1 and HNF3 proteins was never observed. To rule out the possibility that the band corresponding to HNF1 could mask the third complex, gels were submitted to different exposures. No new complex was evidenced. In conclusion, the two proteins are the sole molecules able to bind the specific site, but not simultaneously.

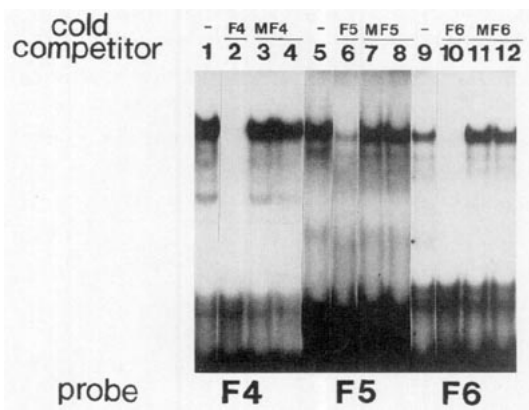

FIG. 5. Mutated HNF3 binding sequences do not compete for the HNF3 factor. *In vitro*-translated HNF3 proteins were incubated with 1 ng of each ^{32}P-labeled oligonucleotide probe F4, F5, or F6; lanes 1, 5, and 9, no competitor; lanes 2, 6, and 10, 100-fold molar excess of cold wild-type oligonucleotide, for F4, F5, and F6, respectively; lanes 3, 7, and 11, 20-fold molar excess; and lanes 4, 8, and 12, 100-fold molar excess of cold mutated oligonucleotides, for MF4, MF5, and MF6, respectively. The sequences of each mutated oligonucleotide are described in Table 1.

To analyze competition between each protein reacting with its corresponding site, they were both incubated at different concentrations (Fig. 6A). At first, the amount of HNF3 was kept constant and that of HNF1 was increased (Fig. 6A, lanes 2–6). Whenever HNF1 was present at low levels, two complexes were detected, but when HNF1 was in excess, the HNF3 complex disappeared (Fig. 6A, lane 6). Second, the quantity of HNF1 was kept constant and that of HNF3 was increased (Fig. 6A, lanes 8–12). Like before, a second complex appeared, and two complexes were always characterized (Fig. 6A, lanes 10–12). In contrast with the results obtained previously for HNF3 studied at a constant level (Fig. 6A, lanes 1–6), HNF1 was not displaced by an excess of the other protein (Fig. 6A, lanes 11 and 12). No supershift corresponding to an association of both factors was detected even in an excess of HNF3. We conclude from these results that both factors are exclusive for their binding to the DNA site. However, HNF1 seems to show a higher affinity for the probe than HNF3, since the former was able to displace HNF3 from the DNA but the opposite could not occur.

To check whether there was steric hindrance between both factors, the two binding sites were separated by 5 and 10 nucleotides in a construct, leading to the forma-

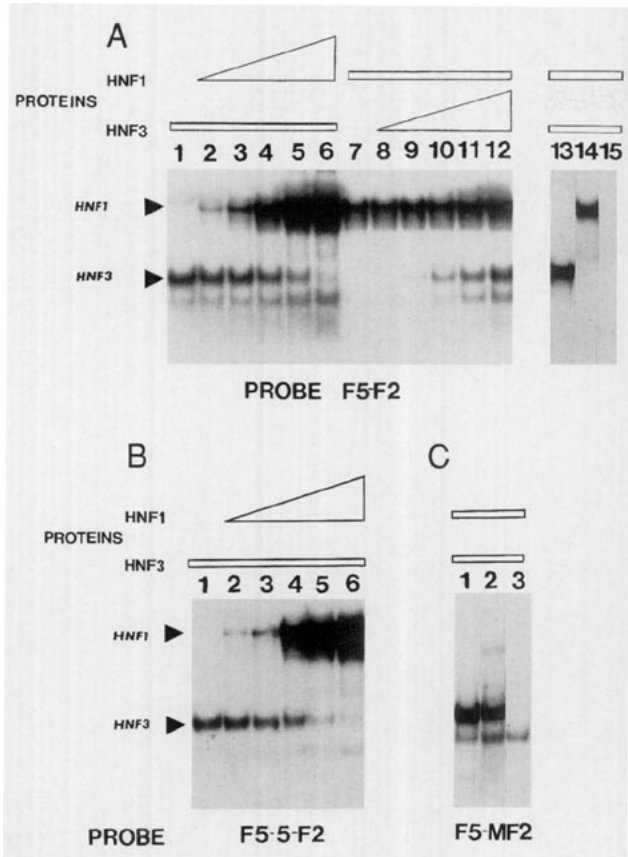

FIG. 6. Inhibition of binding of the HNF3 protein by HNF1 protein on the same DNA fragment. (A) Various proportions of *in vitro*-translated HNF3 and HNF1 proteins were incubated with 1 ng of the ^{32}P-labeled F5-F2 probe. The two proteins, HNF1 and HNF3, were estimated at the same concentration. In lanes 1–6, the HNF3 protein quantity (2 μl) was kept constant, while the HNF1 protein was added under increasing volumes (0, 0.25, 0.5, 1, 2, 4 μl). In lanes 7–12, 1 μl of the HNF1 protein and 0, 0.25, 0.5, 1, 2, 4 μl of the HNF3 protein were added. Lanes 13–15, control experiments with 100-fold molar excesses of cold competitor oligonucleotides F2 (lane 13), F5 (lane 14), and F5-F2 (lane 15) were added, respectively, in the presence of 2 μl of the HNF3 and 1 μl of the HNF1 protein. Arrows point to the position of each complexed binding protein. The band migrating slightly faster than HNF3 is non-specific. (B) Same experiment as for lanes 1–6 in A except that the probe was F5-5-F2 with five nucleotides inserted at the nt 2274, between the HNF3 and the HNF1 binding sites. The same profile was observed with an insertion of 10 nt. (C) Control experiments with the F5-MF2 probe: *in vitro*-translated HNF3 and HNF1 proteins were incubated with 1 ng of ^{32}P-labeled oligonucleotide F5-MF2. This oligonucleotide is identical to F5-F2 except that the HNF1 binding sequence (F2) was mutated (MF2). Lane 1, 2 μl of HNF3 protein; lane 2, 2 μl of HNF3 and 4 μl of HNF1 proteins; lane 3, 4 μl of a reticulocyte lysate.

tion of an additional half turn and one turn in the DNA helix. Therefore a same level of the HNF3 protein (with the lowest affinity) was analyzed in the presence of increasing amounts of HNF1 and conversely. This inclusion of oligonucleotides did not modify the results, indicating that competitive inhibition was not due to steric hindrance. The results are shown only for increasing amounts of the HNF1 protein with the insertion of five nucleotides (Fig. 6B, lanes 1–6).

In order to confirm that an excess of HNF1 added in the solution was not able to displace HNF3, an oligonucleotide involving the F5 site (HNF3) and the mutated HNF1 site (MF2) was studied with both HNF3 and HNF1 proteins. As shown in Fig. 6C, an excess of HNF1 did not modify the binding of HNF3 (Fig. 6C, lanes 1 and 2). Thus, the displacement of HNF3 from its binding site was effectively due to the binding of HNF1 on the same fragment of DNA.

To understand whether this phenomenon was related to the nucleotide sequence of the duck HNF3 binding site (ACTATTGATGT), in which two nucleotides (2nd and 10th position) differed from the consensus sequence (A(A/T)T(A/G)TT(G/T)(A/G)(T/C)T(T/C), we changed the F5 site into the reference HNF3 binding site (TTR: ATTATTGACTT) (Table 2). The oligonucleotide (TTR-F2) containing the strong HNF3 binding site, located at the same distance of the duck HNF1 binding sequence was synthesized (Table 1). Shift experiments by competition were carried out with both HNF1 and HNF3 proteins synthesized *in vitro*. Each protein only bound the corresponding probe (Fig. 7A, lanes 1 and 7), but when equivalent quantities were incubated, a supershift occurred (Fig. 7A, lanes 4 and 10). Thus, both factors could bind simultaneously on the same DNA fragment; moreover, the increase of HNF1 protein in the presence of a constant level of HNF3 or the increase of HNF3 protein in the presence of a constant level of HNF1 enhanced the amount of the slowly migrating complex (Fig. 7A, lanes 5, 6 and 11, 12). This complex was detected even with the lowest amount of HNF1 (Fig. 7A, lane 3) or HNF3 (Fig. 7A, lane 9). Thus disappearance of the HNF3 factor in the presence of an excess of HNF1 (Fig. 7A, lane 6) could be due to sequestration of the HNF3 protein in the HNF1/HNF3 complex rather than the result of a direct competition between both of these factors. As a control of the binding specificity, cold F2, F5, TTR, or TTR-F2 oligonucleotides were added to a mixture of equivalent amounts of HNF1 and HNF3 proteins (as seen in Fig. 7A, lanes 4 and 10). The HNF1 and HNF1/HNF3 bands disappeared in the presence of the F2 competitor (Fig. 7B, lane 1); the HNF3 and HNF1/HNF3 bands disappeared completely in the presence of the TTR oligonucleotide (Fig. 7B, lane 2) and only partially in the presence of the F5 oligonucleotide (Fig. 7B, lane 3), confirming the weaker affinity of the HNF3 for its DHBV F5 site. Furthermore, the three complexes disappeared in the presence of the TTR-F2 oligonucleotide (Fig. 7B, lane 4).

DISCUSSION

A new HNF3 binding site was identified in the DHBV enhancer. This site appears to be located 20 nucleotides upstream from the HNF1 binding site. We also confirm the existence of two other HNF3 binding sequences recently described (Liu *et al.*, 1994). All these sequences share a relative identity with the 11 nucleotides involved

TABLE 1

Oligonucleotides Used in the Experiments

HNF3	
F4 (2210–2229)	5′ *TCGAC*AGCGCAGTGTTTGCTTTTTC*CGCGGG* 3′
MF4	5′ GGGAGCGCAGTGATATCTTTTTC3′
F5 (2251–2278)	5′ *TCGAC*TCAGGAACTATTGATGTCTTGTTTAGCCGCGGG3′
MF5	5′CAGGAACTATGCATGTCTTG3′
F6 (2316–2340)	5′ *TCGAC*AACTTTTGTTTGCCATAAGCGTTACCGCGGG 3′
MF6	5′ AACTTTTGTTATCCATAAGC
TG3T (HBV)	5′*TCGAC*GTCAGCAAACACTTGGCACAGACC 3′
TTR (transthyretin)	5′ *AGCTT*TCTGATTATTGACTTAGTCAAC 3′
HNF1	
F2 (2272–2295)	5′ *TCGAC*TTTAGCCAAGATAATGATTAAACC*GCGGG3′*
MF2	5′GCCAAGATATCGAGTAAACC3′
HNF3/HNF1	
F5-F2₂ (2257–2295)	5′*AGCTT***ACTATTGATGT**CTTGTTTAGCCA**AGATAATGATTAAAC***CGCGGG*** 3′
	HNF3 *HNF1*
F5-5-F2₂ (2257–2274–5T–2275–2295)	5′*AGCTT***ACTATTGATGT**CTTGTTTTTTTTAGCCA**AGATAATGATTAAAC***CG3′***
	HNF3 *HNF1*
F5-MF2₂ (2257–2295)	5′*AGCTT***ACTATTGATGT**CTTGTTTAGCCAAGATATCGAGTAAACC*GCGGG3′*
	HNF3
TTR-F2₂ (TTR–2275–2295)	5′*AGCTT*TCTG**ATTATTGACTT**AGTCAACAGCCA**AGATAATGATTAAAC***CGCGGG3′***
	HNF3 *HNF1*

Note. Each oligonucleotide is represented as a single-strand. The name of the oligonucleotide is followed by the number of the nucleotides (in parentheses) corresponding to the DHBV enhancer. The bases changed are underlined in the mutated oligonucleotides. The additional nucleotides used as restriction sites for cloning are showed in italics and the HNF3 and HNF1 sites are indicated in bold characters.

in the strong HNF3 consensus site defined by Overdier *et al.* (1994) (Table 2).

A first HNF3 sequence located in the 5′ part of the enhancer (F4) has one nucleotide differing from the consensus sequence (G instead of A/T at the 2nd position). Another HNF3 sequence is located in the 3′ part of the DHBV enhancer (F6) differing at positions 1, 10, and 11 from the consensus site. The new site described here (F5) has two mismatches comparatively to the consensus site, a C instead of A/T at the second position, and a G instead of T at the 10th position. This new site is situated near the HNF1 site. No correlation was found between the activity of the three HNF3 DHBV enhancers and the number of mismatches in their binding sequences. The site F4, with one nucleotide differing from the consensus sequence, contributes for 60% of the enhancing activity; the site F6 with three mismatches contributes for 80%. Liu *et al.* (1994) found, respectively, 70 and 60% of the enhancing activity for F4 and F6 in chicken hepatoma cells. Our results are in good agreement with these numbers. The site F5 with two mismatches contributes for 20% of the enhancing activity. This result shows that all the sites are involved and cooperate in the enhancing activity.

One striking observation is the presence of two nucleotides changes in the F5 site at positions 2 and 10. These changes correspond to other mismatches already described for other HNF3 sites. Indeed the HNF3 site described as enhancer for the human α-1-microglobulin/bikunin precursor has four mismatches, two of which were at the same positions 2 and 10 (Rouet *et al.*, 1995); similarly, the binding of HNF3 to this site remained low. Another HNF3 site near the NF1/CTF factor in the liver-specific enhancer of the serum albumin gene has been described with a total of three mismatches, two of which (2 and 10) were always at the same position (Jackson *et al.*, 1993); a competitive inhibition of transcriptional activation between the HNF3 and the NF1/CTF factors was also observed. Thus, the nucleotides at the consensus sites 2 and 10 seem to be essential to modulate the strength of the binding of the HNF3 protein to its site. In contrast, when affecting the "core" of the consensus HNF3 site (positions 3 to 8) the binding was totally abolished (Fig. 5).

The interplay between the HNF3 and the HNF1 factors in the DHBV enhancer was investigated. In gel shift experiments with the DNA fragment bearing both HNF3 and HNF1, the retarded band corresponding to the binding of these fragments was observed, but a supershift corresponding to both factors bound to the DNA fragment never occurred (Fig. 6A, lanes 1–12). In contrast, even with very low quantities of both HNF1 and HNF3 proteins, this supershift was detected with the TTR-F2 probe (Fig. 7A, lanes 3 and 9). To rule out the possibility that HNF1

could inhibit HNF3 binding by lack of room for both proteins competing for their close DNA sites, a space of 5 or 10 nucleotides (a half or one turn of DNA) between the two sites was added. Since no supershift appeared with such an adjunction we can conclude that no sterical hindrance between HNF1 and HNF3 occurred. HNF1 displayed a stronger affinity for its site than HNF3 since this protein could not bind when an excess of the HNF1 protein was added, whereas HNF1 could bind in excess of HNF3. When the F5 sequence was replaced by the HNF3 site corresponding to the TTR sequence, a supershift corresponding to the binding of HNF1 associated to HNF3 was observed. Thus, the TTR sequence in the DHBV enhancer allowed cofixation of the HNF3 and HNF1 binding factors. A difference of two bases (at the 2nd and 10th position) between the DHBV and the TTR sequence induced a modulating capacity between the HNF1 and HNF3 factors. One hypothesis could be that the binding of the HNF1 protein could bend the DNA so that HNF3 no longer binds to its weak site. However, with a strong binding site (TTR) the binding of HNF3 is not modulated by the binding of HNF1. In the context of the whole enhancer, this F5 site still accounts for 20% of the activity. It is possible that HNF3 could be stabilized by other factors in the enhancer. Furthermore, only 20% of the activity (Fig. 4) is far below the 60 to 80% provided by the other factors. The interaction between both factors was analyzed at the transcriptional level by using HNF1 and HNF3 expression vectors transfected in HepG2 cells. However, regulation of the expression of the complete enhancer appeared to be working at too high a level to characterize differences in the enhancing activity.

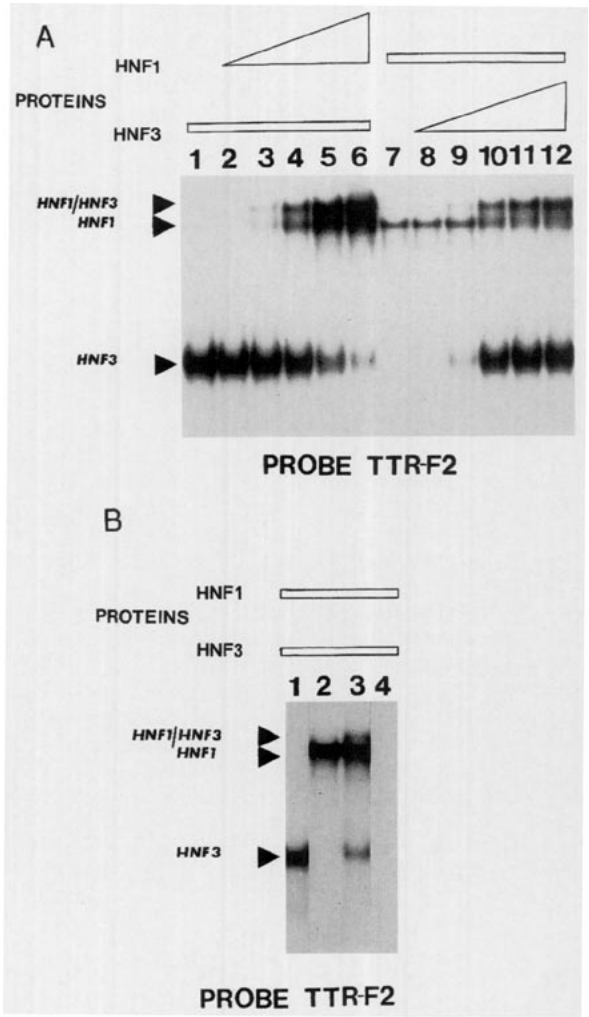

FIG. 7. Binding of HNF3 and HNF1 proteins on the TTR-F2 DNA fragment. Various amounts of *in vitro*-translated HNF3 and HNF1 proteins were incubated with 1 ng of the ^{32}P-labeled TTR-F2 probe. This fragment was identical to the F5-F2 probe used in Fig. 6A except that the binding sequence of the DHBV enhancer F5 (HNF3) was converted here into a consensus site (TTR). (A) Lanes 1–12 same indications as in Fig. 6A. (B) Control competition experiment. Lane 1, 100-fold molar excess of F2 DNA; lane 2, 100-fold molar excess of TTR; lane 3, 100-fold molar excess of the F5 oligonucleotide; and lane 4, 100-fold molar excess of the TTR-F2 DNA cold competitor. Each DNA sequence was added to the mixture containing 1 μl of each of the *in vitro*-translated HNF1 and HNF3 proteins. Arrows point to the position of each complexed binding protein band. Note the supershift band (HNF1/HNF3).

TABLE 2

Comparison between the DHBV HNF3 Binding Sites and the Consensus Sequence

	1	2	3	4	5	6	7	8	9	10	11
Consensus	A	(A/T)	T	(A/G)	T	T	(G/T)	(A/G)	(T/C)	T	(T/C)
F4	A	*G*	T	G	T	T	T	G	C	T	T
F5	A	*C*	T	A	T	T	G	A	T	*G*	T
F6	*T*	T	T	G	T	T	T	G	C	*C*	*A*
TTR	A	T	T	A	T	T	G	A	C	T	T

Note. The consensus sequence defined by Overdier *et al.* (1994) was compared to the F4, F5, and F6 sequences, as well as to the HNF3 binding site in the transthyretin promoter (TTR). Letters in italics correspond to nucleotides differing from the consensus sequence.

Some interactions between other hepatic-specific transcriptional factors have been recently described. In the apolipoprotein B enhancer, HNF4 and C/EBP factors showed cooperation while binding to the DNA (Metzger *et al.*, 1993). In the albumin enhancer, HNF3 was inhibited by the NF1/CTF factor (Jackson *et al.*, 1993). In the human α-1-microglobulin/bikunin precursor gene, HNF3 had a positive effect on the activity of HNF1 (Rouet *et al.*, 1995). In the aldolase B promoter, the sites HNF1 and HNF3 overlapped and competed for the same site (Gregori *et al.*, 1993).

To conclude, the HNF3 and HNF1 factors interplay described here is a new example of a strong HNF1 factor inhibiting the binding of a weaker HNF3 factor on their respective nonoverlapping DNA sites on the DHBV enhancer.

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