Hepatocyte nuclear factor-6 stimulates transcription of the α-fetoprotein gene and synergizes with the retinoic-acid-receptor-related orphan receptor α-4

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The rat α-fetoprotein (*afp*) gene is controlled by three enhancers whose function depends on their interaction with liver-enriched transcription factors. The *afp* enhancer III, located at −6 kb, is composed of three regions that act in synergy. Two of these regions, called s1 and s2, contain a putative binding site for hepatocyte nuclear factor-6 (HNF-6). This factor is the prototype of the ONECUT family of cut-homoeodomain proteins and is a known regulator of liver gene expression in adults and during development. We show here that the two splicing isoforms of HNF-6 bind to a site in the s1 region and in the s2 region. The core sequence of the s1 site corresponds to none of the known HNF-6 binding sites. Nevertheless, the binding properties of the s1 site are identical with those of the s2 site and of previously characterized HNF-6 binding sequences. The HNF-6 consensus should therefore be rewritten as DRRTCVATND. Binding of HNF-6 to the s1 and s2 sites requires both the cut and the homoeo domains, is co-operative and induces DNA bending. HNF-6 strongly stimulates the activity of the *afp* enhancer III in transient transfection experiments. This effect requires the stereo-specific alignment of the two HNF-6 sites. Moreover, HNF-6 stimulates the enhancer in synergy with the retinoic-acid-receptor-related orphan receptor α (RORα), which binds to a neighbouring site in the s1 region. Thus expression of the *afp* gene requires functional interactions between HNF-6 molecules and between HNF-6 and RORα.

Key words: liver differentiation, transcription factor.

INTRODUCTION

Cell growth and differentiation require a tight regulation of gene expression. This regulation is exerted primarily at the level of transcription initiation by the combinatorial action of transcription factors. The ability of transcription factors to regulate gene expression depends on their affinity for their DNA recognition sequences and on their interactions with other transcription factors and components of the basal transcription machinery [1–3]. DNA topology and chromatin structure also play also a role in these processes [4–6].

Hepatic differentiation involves the action of liver-enriched transcription factors that act in combination with more widely expressed transcription factors (see, for reviews, [7–10]). Such liver-enriched transcription factors include the homoeodomain proteins of the hepatocyte nuclear factor-1 (HNF-1) family, two members of the zinc-finger nuclear orphan receptor family (HNF-4 and fetoprotein transcription factor), the winged helix forkhead proteins HNF-3/FOXA, the leucine-zipper factors related to CCAAT/enhancer binding protein and D-binding protein, some of the signal transducers and activators of transcription (STAT-3 and -5b) and the members of the ONECUT class homoeoproteins whose prototype is HNF-6 (see, for reviews, [8,9,11]). These factors act in networks, since, for instance, HNF-6 controls the *hnf-1β*, *hnf-3β* and *hnf-4α* genes, while HNF-4α in turn controls the *hnf6* gene [12–15].

The α-fetoprotein gene (*afp*) has been extensively studied to understand how gene expression is controlled in liver and how it is modulated during development and cancerogenesis. The *afp* gene is expressed in the visceral endoderm. During liver development its expression starts in the presumptive territory of the liver in the endoderm and is maintained in liver until birth. *afp* expression resumes in liver regeneration and cancerogenesis. The *afp* gene is essentially controlled at the transcriptional level. The *cis*-regulatory elements that are important for specific expression in liver and hepatoma cells are located within 7 kb upstream of the transcription initiation site. In the rat, three enhancers have been characterized in this region: EI (−2.4 kb to −2.8 kb), EII (−3.9 kb to −4.5 kb) and EIII (−5.7 kb to −6.1 kb) (see, for reviews, [16–19]). Enhancer III is the most potent of the three enhancers in transfection experiments [20–23], and it can drive transgene expression in the liver of transgenic mice [22,23]. This enhancer contains three contiguous regions that act in synergy [20]. The s1 region, which is well conserved in the mouse, is essential for liver-specific expression in transgenic mice [23]. It binds several nuclear proteins [20], which include the retinoic-acid-receptor-related orphan receptor α (RORα/NR1F1 [24,25]. RORα is known to control differentiation and hormone action in several tissues [26]. It binds as a monomer to the AGGTCA motif in the s1 region of the *afp* gene enhancer III and stimulates its activity [25]. The s2 region binds HNF-3 [20,21]. It is also a putative target for HNF-6

Abbreviations used: AFP, α-fetoprotein; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic-mobility-shift assay; HNF-3, hepatocyte nuclear factor 3; HNF-6, hepatocyte nuclear factor 6; rHNF-6, recombinant HNF-6; RORα, retinoic acid receptor-related orphan receptor α; ttr, transthyretin.

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[27], which is co-expressed with the *afp* gene in the early steps of development [15]. This led us to investigate the role of this transcription factor in the activity of the *afp* enhancer. We show here that both the s1 and s2 regions contain a binding site for HNF-6 and that these sites co-operate in the binding reaction. We also show that HNF-6 bends DNA and that it stimulates the activity of the *afp* enhancer in synergy with RORα.

EXPERIMENTAL

Oligonucleotides and probes

Wild-type or mutated double-stranded oligonucleotides spanning the s1 or s2 region of the rat *afp* gene enhancer III have been described [20] and are schematized in Figure 1(A). DNA fragments spanning the wild-type or mutated s1-s2 region were obtained by PCR amplification of the corresponding chloramphenicol acetyltransferase (CAT) plasmids. Wild-type or mutated probes were labelled with [α-^{32}P]dATP (3000 Ci/mmol; Amersham) and Klenow polymerase or with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) and T_4 DNA polynucleotide kinase. They were recovered after purification by Chroma Spin™ columns (ClonTech Inc.).

Plasmids

Expression vectors

The empty pECE72 vector and pECE-HNF6α, pECE-HNF6β, pECE-HNF6αF48W, pECE-HNF6αM50H, pECE-HNF6α F48W + M50H, pECE-HNF6$\alpha\Delta$Ser and pECE-HNF6$\alpha\Delta$Hd vectors used for overexpression experiments and the empty pSP72 vector and pSP-HNF6α, pSP-HNF6β, pSP-HNF6$\alpha\Delta$Hd and pSP-HNF6αCut + Hd vectors used for *in vitro* transcription–translation have been described [28]. pGem-2-CMV-HNF-3α and pGem-2-CMV-HNF-3β [29] were kindly given by Dr. R. H. Costa (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL, U.S.A.). pCMX and pCMX-mRORα4 vectors [30] were kindly provided by Dr. V. Giguère (McGill University Health Center, Montreal, Quebec, Canada).

Plasmids used for circular permutation analysis

These plasmids were constructed from pBend3 [31], which was kindly given by Dr. A. Kolb (Institut Pasteur, Paris, France). The plasmid pBend3-s2 containing the s2 region was constructed by ligating the double-stranded synthetic oligonucleotide s2, 5′-AGTGTACCTTTATTGACTTTGACATATTTC-3′, into the end-filled *Xba*I site of pBend3. To generate circularly permutated labelled DNA fragments, pBend3-s2 was digested with the linker restriction enzymes, made blunt, dephosphorylated by calf intestinal alkaline phosphatase (Promega) and end-labelled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) and T_4 DNA polynucleotide kinase. The probes were purified by PAGE and recovered by electroelution followed by phenol extraction and ethanol precipitation. The gels for the electrophoretic-mobility-shift assay (EMSA) were analysed in a quantitative manner with the Instant Imager (Packard) to measure the distance of migration of the complexes and of the free probes.

Reporter constructs

The pBLCAT6 vector which contains the bacterial chloramphenicol acetyltransferase (*cat*) gene was used to make reporter constructs bearing the s1 region or the s1-s2 region of the rat *afp* gene enhancer III in front of the cytomegalovirus (CMV) promoter. pCMV-CAT was first constructed by insertion of the CMV promoter, obtained by *Bgl*II digestion of pHNF-6/TTR (6x)-TATA-luc construct [28], into *Bgl*II sites of pBLCAT6. The orientation of the CMV promoter was determined by sequencing.

The plasmid s1-CMV-CAT was constructed by ligating the double-stranded synthetic oligonucleotide s1 into the end-filled *Sal*I site of CMV-CAT. To destroy in this reporter construct both the HNF-6 and the RORα binding sites, the double-stranded oligonucleotide s1MM′ 5′-TGCTGTAACTCTCCTTAAGCTATATCGCGATGTTCTAGTG-3′ was cloned into the end-filled *Sal*I site of CMV-CAT. The orientation of the inserted oligonucleotide in s1-CMV-CAT and in s1MM′-CMV-CAT was determined by DNA sequencing. The s2-s1-CMV-CAT construct was obtained by excising a DNA fragment corresponding to the rat *afp* gene enhancer III from pUC19-E3 [20] with *Bam*HI and *Sph*I and cloning it into the *Bam*HI/*Sph*I sites of CMV-CAT. Mutants of this construct in which the HNF-6 binding site is destroyed in either region s1 (s2-s1M-CMV-CAT; ATATCGCGATGTT), or region s2 (s2M-s1-CMV-CAT; TTATTCAATTTGA) were obtained by site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis Kit from Stratagene and appropriate sets of oligonucleotides. The same mutagenesis strategy was also used to introduce 5 or 10 bp between the s1 region and the s2 region in s2-s1-CMV-CAT. The s2-5-s1-CMV-CAT construct, contains a 5 bp insertion (-TCTAGTCTAGAGTACCTT-), whereas the s2-10-s1-CMV-CAT construct contains a 10 bp insertion (-TCTAGTCTAGATATCCGTACCTT-). All plasmid constructs were verified by DNA sequencing and purified using Concert™ High Purity Plasmid Purification Systems (Life Technologies). At least two different preparations of each plasmid were used in the transfection experiments.

Production of recombinant proteins

Production of recombinant HNF-6 and HNF-3 proteins in COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium ('DMEM/Nutriment mixture F-12 with Glutamax I'; Invitrogen Life Technologies, Paisley, Scotland, U.K.) supplemented with 10% (v/v) fetal-calf serum. A total of 2.5×10^5 cells were plated in 10-cm-diameter dishes 24 h prior to transfection. The cells were transfected by the calcium phosphate co-precipitation method [32] using 5 μg of expression vector for HNF-6 or HNF-3. Following overnight incubation, the cells were washed, fed with fresh medium and incubated for an additional 24 h. The cells were washed with PBS before being harvested in 1 ml of TEN-buffer [40 mM Tris/HCl (pH 7.5)/1 mM EDTA/150 mM NaCl]. The cells were pelleted and resuspended in 200 μl of lysis buffer [50 mM Tris/HCl (pH 7.9)/500 mM KCl/20% (v/v) glycerol/0.5 mM EDTA/1 mM PMSF/0.1% Nonidet P40/1 mM dithiothreitol/2.5 μg/ml leupeptin]. After three freeze–thaw cycles and centrifugation, the supernatants were collected and protein concentrations were determined by the Bradford method with bovine immunoglobulins (Bio-Rad) as standards.

In vitro synthesis of recombinant proteins

The deletant HNF6α(cut + hd) was obtained by *in vitro* transcription–translation, using the TnT® Sp6 Coupled Reticulocyte Lysate System from Promega. These experiments were carried out with 1 μg of the corresponding plasmid in 50 μl of lysate according to the supplier's instructions.

EMSA

Binding reactions were carried out in 20 μl of binding buffer [10 mM Tris/HCl (pH 8)/50 mM KCl/7% (v/v) glycerol/1 mM dithiothreitol] containing 4 μg of poly(dI-dC) and 10000 c.p.m.

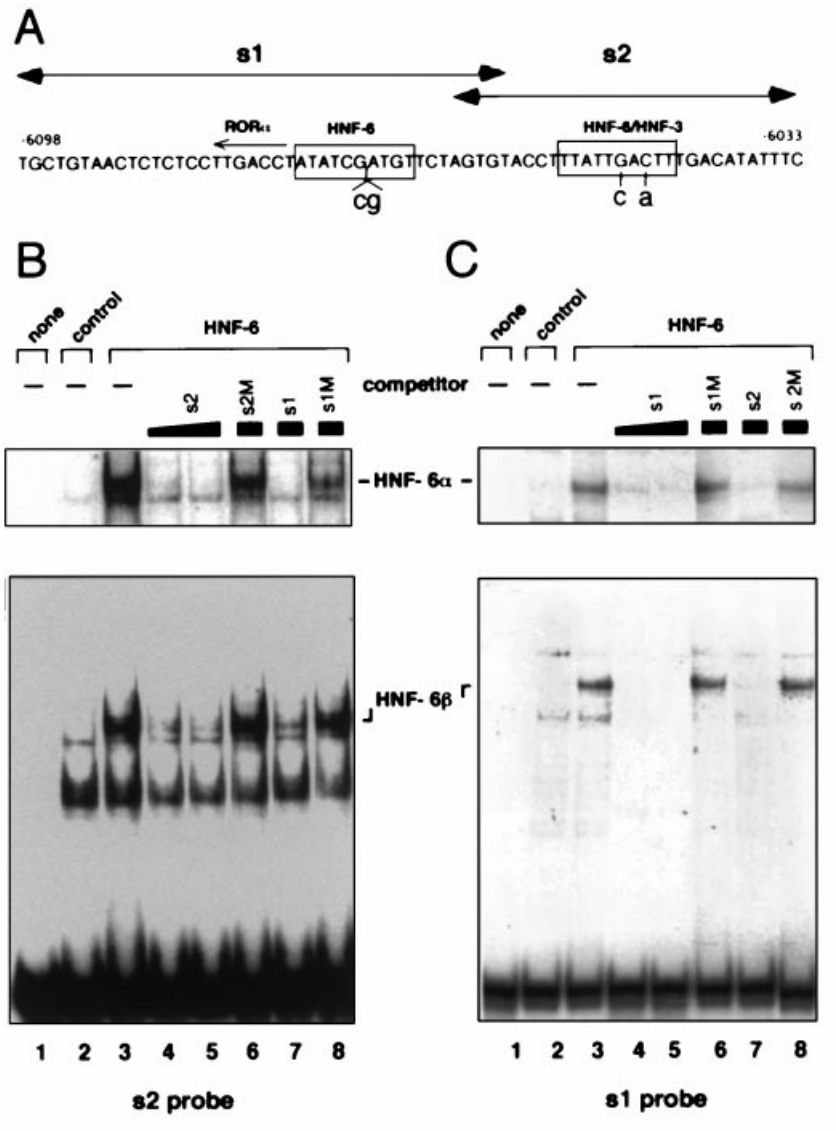

Figure 1 HNF-6α and HNF-6β bind specifically to the s1 and s2 regions of enhancer III

(**A**) Oligonucleotide sequence of the s1 and s2 regions of afp enhancer III. As shown under the bi-directional arrows, double-stranded oligonucleotides were designed to span the wild-type s1 and s2 regions of the rat *afp* gene enhancer III. Only the nucleotide sequence of the upper strand is shown. Numbering is from the transcription start site. The nucleotide sequences corresponding to the HNF-6 binding sites are boxed. Lower-case letters indicate the mutations introduced in the s1 region (s1M) and in the s2 region (s2M). (**B**) Binding of HNF-6 to the s2 region. EMSAs were performed with the s2 probe and extracts from COS-7 cells transfected with the empty pECE72 vector (lane 2) or with the HNF-6α or HNF-6β expression vector. Lane 1, s2 probe alone; lanes 3–8, s2 probe with a fixed amount of rHNF-6α or HNF-6β without (lane 3) or with 50-fold (lane 4) and 100-fold (lane 5) molar excess of s2 oligonucleotide or 100-fold molar of the other oligonucleotides indicated (lanes 6–8). (**C**) Binding of HNF-6 to the s1 region: EMSA were performed with the s1 probe and extracts from COS-7 cells under the same conditions as in (**B**).

of the ^{32}P-labelled probe and 5 μg of COS-7 protein extracts or 5 μl of *in vitro* transcription–translation reaction product. For competition experiments, unlabelled oligonucleotides were first mixed with the ^{32}P-labelled probe before adding proteins. After 20 min at 4 °C, binding reaction mixtures were resolved on non-denaturing polyacrylamide gels (acrylamide/bisacrylamide ratio 30:1) at 6 % for oligonucleotide probes, 5 % for the s1-s2 probes and 5 or 8 % for permutated probes. Gels were run at 200 V and at 4 °C in 0.25 × TBE buffer [where 10 × TBE is Tris/borate/EDTA at concentrations of 121.1 g/l, 61.8 g/l and 7.44 g/l respectively (pH 8.3)]. Gels were fixed in 20 % (v/v) ethanol/10 % (v/v) acetic acid, dried and exposed to Hyper Film (Amersham). Quantitative measurements of the protein–DNA complexes were done on an Instant Imager (Packard).

Culture of HepG2 cells, transfection and CAT assays

The culture and the transfection of human hepatoma cells HepG2 by the calcium phosphate procedure and the determination of CAT activities were performed as described [32]. Routinely, 2 μg of CAT plasmid and 5 or 10 μg of expression vector were used. Care was taken to keep constant the amount of DNA with empty vectors. The results are given as the means ±

S.E.M. for five to ten independent transfection experiments, usually run in duplicate.

RESULTS AND DISCUSSION

HNF-6 binds to two regions of the *afp* enhancer III

The consensus sequence for the HNF-6 binding sites is DRRTCMATND [28]. The sequence of the *afp* enhancer III contains a putative site not only in the s2 region [27], but also in the s1 region (Figure 1A). To determine whether these two sites actually bind HNF-6, oligonucleotide probes were incubated with extracts from cells transfected with HNF-6 expression vectors and binding was tested by EMSA. Two splicing isoforms of HNF-6 have been described. The length of the spacer region between the cut domain and the homoeodomain is 26 amino acids longer in HNF-6β than in HNF-6α, and this modulates their DNA-binding affinity [28]. Therefore the two isoforms were tested for binding to the *afp* enhancer.

As Figure 1(B) shows, recombinant HNF-6α (rHNF-6α) or HNF-6β (rHNF-6β) bound to the probe corresponding to the s2 region (lane 3). Binding was specific, since it was not observed with excess of unlabelled s2 oligonucleotide (lanes 4–5), but was still observed with excess of unlabelled oligonucleotide mutated in the HNF-6 consensus (lane 6). HNF-6 binding to the s2 probe was abolished with excess of unlabelled s1 oligonucleotide, but not of s1 oligonucleotide mutated in the HNF-6 consensus (lanes 7–8). This indicated that the s1 region does bind HNF-6. This was tested directly with the labelled s1 probe (Figure 1C). Binding of HNF-6α and HNF-6β was detected, and it was prevented by excess of unlabelled s1 or s2 oligonucleotide, but not with the mutated ones (Figure 1C, lanes 4–8). We concluded that HNF-6 binds to the s1 and s2 regions of enhancer III. These HNF-6 sites are perfectly conserved in the mouse [23].

Interestingly, the site in the s1 region overlaps with the *ClaI* site which is indispensable for directing activity of enhancer III in liver of transgenic mice [23]. It contains a guanosine residue (G) at position 6 of the consensus (ATATCGATGT), in contrast with the known HNF-6 sites which contain an adenosine (A) or a cytosine (C) residue at this position [27,33]. The HNF-6 binding site consensus should therefore be rewritten as DRRTCVATND. Since the HNF-6 site in the s2 region also corresponds to an HNF-3 site [20,27], we attempted to verify whether HNF-3 binds to the HNF-6 site in the s1 region. Indeed, an ATNGAT motif, like that found in the HNF-6 binding site of the s1 region, is often present in HNF-3 binding sites [34]. We performed EMSA with the s1 and s2 probes and rHNF3α or HNF-3β. We found that HNF-3 bound to the s2 probe as expected (Figure 2C, lanes 2 and 3), but not to the s1 probe (Figure 2B, lanes 2 and 3), indicating that the HNF-6 site in region s1 is not a common target for HNF-3.

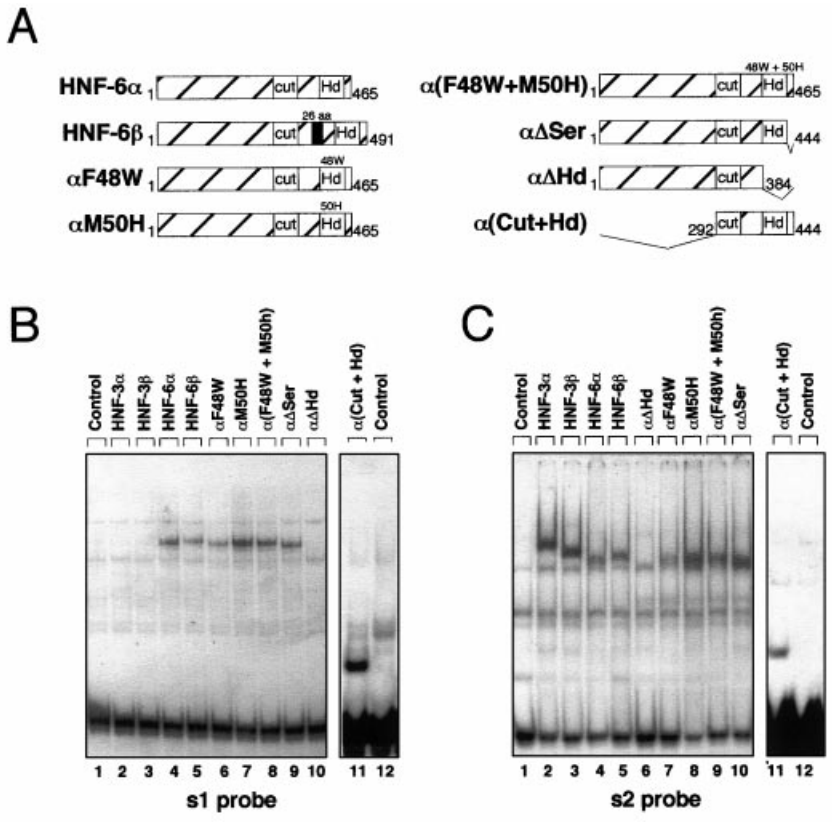

Figure 2 Similar modes of binding of HNF-6 to its two sites in the *afp* enhancer III

(**A**) Schematic representation of wild-type HNF-6α, HNF-6β (with the 26-amino-acid insert) and of the HNF-6α mutants (37) used in EMSA. Numbers refer to amino acid positions. (**B**) and (**C**), EMSA reactions with the s1 probe (**B**) or with the s2 probe (**C**) and extracts from COS-7 cells transfected with the empty pECE72 vector (control) or expression vectors for HNF-3 or HNF-6 proteins as indicated above the lanes 1–10. In lanes 11 and 12, EMSA was performed with reticulocyte lysates programmed with the pSpHNF-6αCut + Hd vector and with the empty pSp72 vector as control.

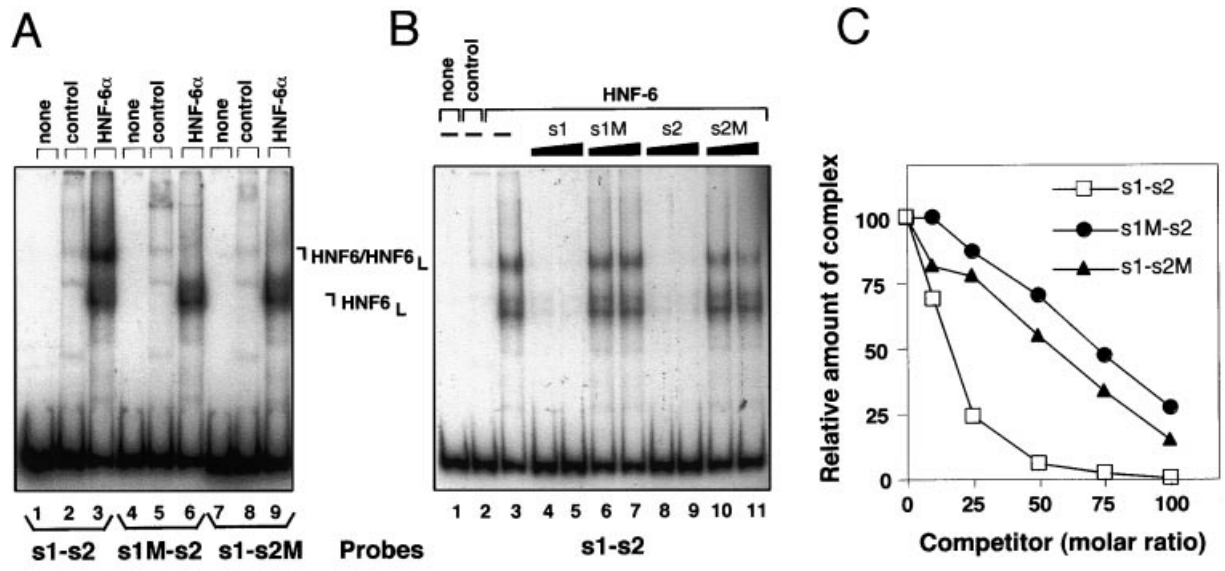

Figure 3 HNF-6 binds to the s1 and s2 regions of enhancer III in a co-operative manner

(**A**) EMSAs were performed with the indicated labelled probe alone (lanes 1, 4 and 7) or with extracts from COS-7 cells transfected with the empty pECE72 vector as control (lanes 2, 5 and 8) or with the HNF-6α expression vector (lanes 3, 6 and 9). (**B**) EMSAs were performed with the labelled s1-s2 probe alone (lane 1) or with extracts from COS-7 cells transfected with the empty pECE72 vector as control (lane 2) or with the HNF-6α expression vector (lanes 3–11). Unlabelled competitor oligonucleotides were included at 100- and 200-fold molar excess in the binding reactions (lanes 4–11) as indicated. (**C**) EMSA were performed with the labelled s1-s2 probe and extracts from COS-7 cells transfected with the HNF-6α expression vector. Different amounts of unlabelled competitor oligonucleotides were included in the binding reactions as indicated. Results are the means for two or three experiments.

Similar modes of binding of HNF-6 to its two sites in the *afp* enhancer III

Two types of HNF-6 binding sites are known: those of the type found in the *transthyretin* (*ttr*) promoter, for which both the cut domain and the homoeodomain are required for DNA binding, and those of the type found in the hnf3β promoter, for which the cut domain is sufficient [28]. To determine to which type the s1 and s2 HNF-6 binding sites belong, we tested a series of HNF-6α mutants by EMSA. The HNF-6α mutant containing only the cut domain plus the homoeodomain [HNF-6α (cut + Hd); Figure 2A] bound to both probes (Figures 2B and 2C, lane 11), but deletion of the homoeodomain (HNF-6αΔHd) abolished binding (Figure 2B, lane 10, and Figure 2C, lane 6). This was not due to lack of synthesis of the mutant protein, since the same extracts from COS-7 cells transfected with HNF-6αΔHd produced in EMSA the expected complex with an hnf3β probe ([28]; results not shown). Deletion of the cut domain also abolished binding to the s1 and s2 probes (results not shown). All these results indicated that the s1 and s2 sites belong to the *ttr* type site.

The s1 and s2 sites also bound other HNF-6 mutants, as expected for *bona fide* HNF-6 sites [28]. Indeed, deletion of the C-terminal serine-rich region did not abolish binding (Figure 2B, lane 9, and Figure 2C, lane 10). The same was true when the two conserved residues of the homoeodomain, namely Phe^{48} and Met^{50}, which are a characteristic of the ONECUT proteins, were mutated into residues that are typical of classical homoeodomains. As shown in Figure 2(B) (lanes 6–8) and Figure 2(C) (lanes 7–9), the simple ($Phe^{48} \rightarrow Trp$ and $Met^{50} \rightarrow His$) or double ($Phe^{48} \rightarrow Trp/Met^{50} \rightarrow His$) mutations did not significantly alter the binding of HNF-6 to the s1 region or s2 region, in keeping with previous work [28]. Thus the specific Phe^{48}/Met^{50} dyad of the HNF-6 homoeodomain is not critical for the binding of HNF-6 to the s1 region and s2 region of the rat *afp* enhancer III.

Taken together, these data indicated that the two binding sites of enhancer III characterized here have similar properties in terms of HNF-6 binding.

HNF-6 binds to the s1 and s2 regions of the *afp* enhancer III in a cooperative manner

The two HNF-6 binding sites described here in enhancer III are 12 bp apart (Figure 1A). To test if binding of HNF-6 to its two sites in the *afp* enhancer is co-operative, we performed EMSA on wild-type or mutated oligonucleotide probes spanning the s1-s2 region (see Figure 1A for the probe sequence). Binding reactions with rHNF-6α and the s1-s2 probe displayed two specific complexes (Figures 3A and 3B, lane 3). As shown in Figure 3(B), the complexes were inhibited by an excess of unlabelled s1 (lanes 4–5) or s2 (lanes 8–9) oligonucleotide, but not by an excess of unlabelled s1M (lanes 6–7) or s2M (lanes 10–11) oligonucleotide in which the HNF-6 binding site is mutated. As shown in Figure 3(A), experiments carried out with the labelled s1M-s2 (lane 6) or s1-s2M (lane 9) probe yielded only the faster-migrating complex (lane 3). Thus the latter complex corresponded to the binding of one HNF-6 molecule to either the s1 or the s2 region, while the more slowly migrating complex corresponded to the simultaneous binding of two HNF-6 molecules to the s1-s2 region. Formation of the slower complex occurred while the free s1-s2 probe was still in excess, suggesting that the binding of HNF-6 to the s1-s2 probe is co-operative. To test this hypothesis, we performed EMSA using the s1-s2 probe and s1-s2, s1M-s2 or s1-s2M oligonucleotide as competitors. These experiments showed that the unlabelled s1-s2 oligonucleotide competed more efficiently than unlabelled mutated oligonucleotide s1M-s2 or s1-s2M (Figure 3C). Moreover, full competition with the unlabelled s1-s2 probe was achieved over less than two logarithmic units of competitor concentration, as expected for positive co-operativity

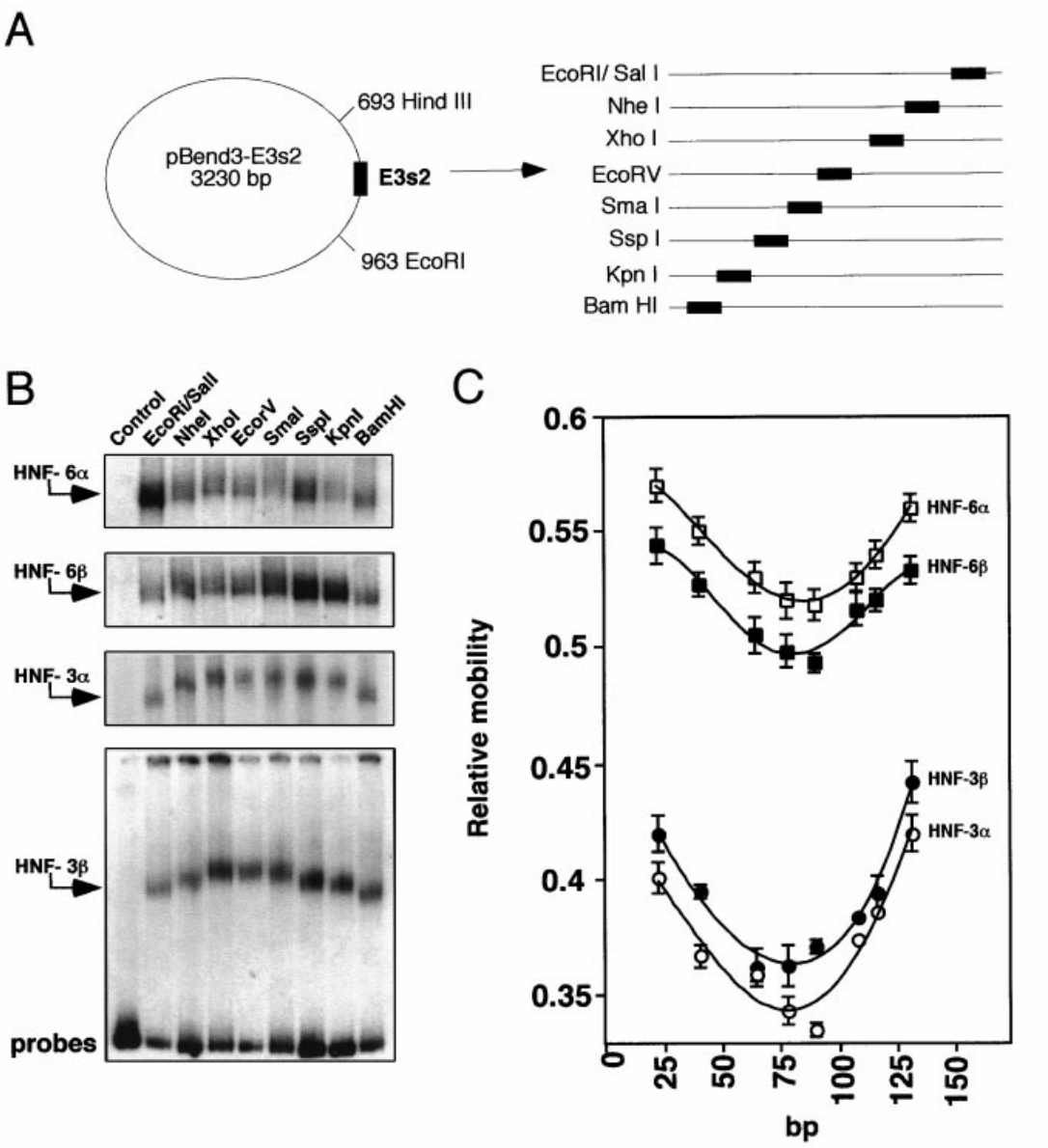

Figure 4 HNF-6 bends DNA

(**A**) Schematic representation of the circularly permutated DNA fragments used to investigate DNA bending. The s2 oligonucleotide was cloned into the *Xba*I site of the circular permutation vector pBend3 to generate pBend3-s2. Digestion of pBend3-s2 with the indicated restriction enzymes generated permutated fragments with the same 155-bp length, but with different location of the HNF-6 site. (**B**) Circular permutation analysis by EMSA with labelled DNA fragments incubated with extracts from COS-7 cells transfected with HNF-6α, HNF-6β, HNF-3α or HNF-3β expression vector. As a control, the binding reaction was performed using the *Eco*RI–*Sal*I-labelled fragment and extracts from COS-7 cells transfected with the empty pECE72 vector. (**C**) Permutation curves. The relative migration of the complexes was plotted as a function of the position of the binding site (measured in bp from the centre of the HNF-6 binding site to the 3′ end of the DNA fragment).

[35]. All these data demonstrated that HNF-6 can bind simultaneously to the s1 region and to the s2 region with similar affinities and that the binding of the two HNF-6 molecules to the s1-s2 region is co-operative.

Binding of HNF-6 induces DNA bending

Binding of proteins to DNA can lead to bending of DNA, and this may contribute to the effect of transcription factors (see, for reviews, [36,37]). While DNA bending by HNF-3 has been demonstrated [38], no information is available concerning the effect of HNF-6 on the curvature of DNA. The fact that both HNF-6 and HNF-3 bind to the s2 region (Figures 2B and 2C, lanes 2 and 3) allowed for a comparison of the curvature they induce upon binding to the same DNA sequence, by doing circular permutation experiments [39]. These experiments are based on the observations that DNA bending retards migration of protein–DNA complexes in non-denaturing gels and that this effect is stronger when bending affects the centre of the DNA fragment. To this end, the s2 region was cloned into the circular permutation vector pBend3 to generate pBend3-s2. Digestion of the vector with various restriction endonucleases released inserts of identical size (155 bp) and base composition, but with different relative position of the s2 binding site (Figure 4A).

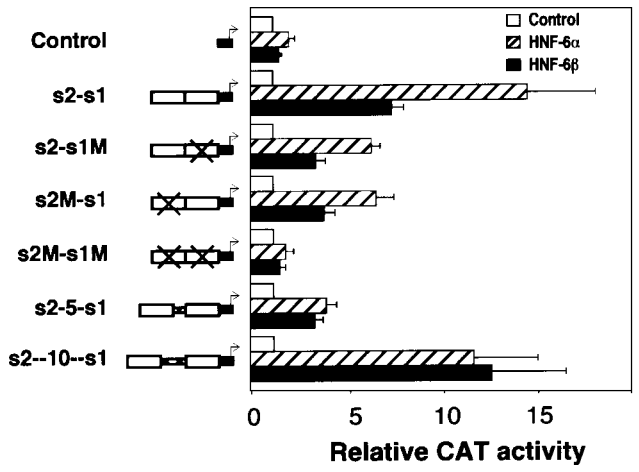

Figure 5 HNF-6α and HNF-6β transactivate enhancer III

HepG2 cells were cotransfected with the CMV-CAT reporter constructs indicated and with the empty pECE-72 vector (control) or the pECE-HNF-6α or pECE-HNF-6β expression vector. CAT activities are expressed relative to the control. Values are means ± S.E.M. for five to ten experiments performed in duplicate.

EMSA showed that rHNF-6α and HNF-6β formed a specific complex with each of the probes (Figure 4B). However, the complexes had different mobilities, the lowest being observed with the complex formed with the *Eco*RV probe in which the s2 site is located in the centre of the DNA probe. The relative mobilities plotted as a function of the position of the HNF-6 site in the probe (permutation curves) indicated that HNF-6 induces DNA bending. The permutation curve obtained with the HNF-6α(Cut + Hd) mutant containing only the cut and homoeodomains was very similar to that of HNF-6α (results not shown), indicating that the DNA-binding domain of HNF-6 was responsible for the bending. The two HNF-6 isoforms induced similar bending of DNA (Figures 4B and 4C), suggesting that the 26-amino-acid spacer between the cut domain and the homoeodomain of HNF-6β does not influence bending. The permutation curves obtained for HNF-3α and HNF-3β showed that these proteins also bend DNA when they bind to the s2 site (Figures 4B and 4C).

The value of the μM/μE ratio, where μM and μE are the relative mobilities of the complexes with DNA fragments in which the binding site is located either in the middle (M) or at the end (E) of the DNA fragment, is inversely correlated to the degree of the induced bending [40]. The μM/μE value calculated for HNF-6α and HNF-6β was 0.913 ± 0.004 ($n = 6$). A smaller value was calculated for HNF-3α (0.743 ± 0.004, $n = 3$) and for HNF-3β (0.781 ± 0.006, $n = 3$). The deduced value of the angle of DNA-bending ($\mu M/\mu E = \cos\alpha/2$, where '$\alpha$' represents the angle of DNA-bending) [40] was about 50° when induced by HNF-6, and about 80° when induced by HNF-3α or HNF-3β. This α value for HNF-3 is in agreement with the permutation analysis that showed that forkhead-related activators, which belong to the same family as HNF-3, induce an angle of bending of 80–90° [41]. Taken together, our data indicated that HNF-6 induces bending of the DNA, but to a smaller extent than HNF-3.

To our knowledge, the present work is the first to compare the bending of the same DNA sequence by two transcription factors belonging to distinct families. Since HNF-6 and HNF-3 induce different bending, the two factors could exert different effects via the same *cis*-acting sequence. For instance, the difference in the curvature induced by HNF-3 or HNF-6 may favor recruitment of different sets of proteins to adjacent DNA regions.

HNF-6 transactivates the *afp* enhancer III

To evaluate the effects of HNF-6α and HNF-6β on enhancer III activity, we used reporter constructs in which the *cat* gene is controlled by the CMV promoter and by wild-type or mutated s1 and s2 regions (Figure 5). These constructs were co-transfected with the HNF-6α or HNF-6β expression vector into HepG2 cells. As shown in Figure 5, HNF-6α and HNF-6β had no activity on the CMV promoter, but strongly stimulated transcription when this promoter was linked to the s1-s2 region. This effect was dependent on the amount of expression vector (results not shown). We then investigated the relative activities of HNF-6α and HNF-6β on each of the two binding sites. Mutation of the HNF-6 site in the s1 region decreased by about 50 % the stimulatory effect of HNF-6α and HNF-6β (Figure 5). The same was true for mutation of the HNF-6 site in the s2 region. The double mutant was unresponsive to HNF-6α or HNF-6β. These results showed that HNF-6 stimulates enhancer III and that each of the two HNF-6 binding sites identified in the enhancer contributes to the same extent to transcriptional stimulation.

To test whether the stereospecific alignment of the two HNF-6 binding sites is critical for the transactivating effect of HNF-6, spacer-length variants of the reporter construct were designed. The insertion of 5 bp (half helical turn of DNA) between the s1 and s2 regions resulted in a strong decrease in the stimulatory effect of HNF-6α and HNF-6β (Figure 5). Insertion of 10 bp (full helical turn) did not affect significantly stimulation by HNF-6 as compared with wild-type constructs. Thus the *trans*-activating effect of HNF-6 on enhancer III appeared to depend upon the orientation of the two HNF-6 sites relative to one another, but not upon their distance. Interestingly, the HNF-6 transactivating effect is optimal in the natural context of the two HNF-6 binding sites in the *afp* enhancer. These observations suggested that direct or indirect protein–protein interactions take place between the two HNF-6 molecules bound to the s1-s2 region of the *afp* enhancer III.

HNF-6 and RORα4 synergize to transactivate the *afp* enhancer III

The HNF-6 binding site in the s1 region is contiguous with the RORα binding site (Figure 1A) [25]. These two sites are perfectly conserved in the rat [20] and the mouse [23]. We therefore attempted to verify whether there is a functional interaction between HNF-6 and RORα4, the predominant isoform of RORα in the liver [42]. Transient co-transfection experiments were performed with HepG2 cells using the s1-CMV-CAT plasmid, in which the *cat* gene is controlled by the CMV promoter and the s1 region. RORα4, HNF-6α or HNF-6β alone stimulated the activity of the s1-CMV-CAT 2.5-, 12- and 7.5-fold respectively (Figure 6). Interestingly, the simultaneous presence of RORα4 and HNF-6α or HNF-6β strongly increased (up to 21–23-fold) the transcriptional activity of the s1 region. These results showed that HNF-6 and RORα4 transactivate in a synergistic manner. As expected, the s1MM′-CMV-CAT construct, in which both the HNF-6 and the RORα4 sites are inactivated by mutations, was unresponsive to HNF-6 and RORα4 (Figure 6). Such a synergistic effect between HNF-6 and RORα4 was not observed when using reporter plasmids carrying only several copies of either HNF-6 (p(HNF6-HNF3β)6x-LUC [28] or RORα4 (p(RORE)x3-TKLUC [30] binding sites (results not shown), showing that HNF-6 and RORα4 both must bind to DNA to exert their synergistic effect.

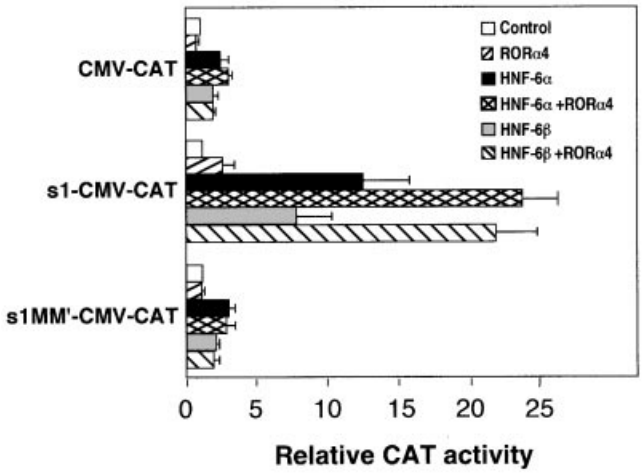

Figure 6 HNF-6 and RORα4 transactivate enhancer III synergistically

HepG2 cells were co-transfected with the CMV-CAT reporter constructs carrying the s1 region (either wild-type or mutated in both the RORα and the HNF-6 sites) and with expression vectors for the recombinant proteins listed. The empty vectors pECE72 and pCMX were used as controls. The total amount of DNA was maintained constant by adding the corresponding empty vectors. Relative CAT activity values are means ± S.E.M. for four to eight experiments performed in duplicate.

The molecular mechanism of the synergy between HNF-6 and RORα4 is not known. One appealing hypothesis is that a co-activator such as p300/CBP, which binds HNF-6 [43] and the E region of RORα [44], is involved in this mechanism. It may also well be that HNF-6 and RORα4 interact directly. Direct interactions between HNF-6 and another member of the nuclear receptor family, namely the glucocorticoid receptor, have been observed [45]. Consistent with the known synergy between RORα and the muscle-specific factor MyoD in the control of muscle differentiation [26], our present findings suggest that the ubiquitously expressed RORα is also an important accessory factor for liver-enriched transcription factors such as HNF-6. On the other hand, HNF-6 is known to act in synergy with the liver-enriched transcription factors HNF-3β to stimulate transcription of the cyp2c12 gene [46] and HNF-1α to stimulate transcription of the HNF4α gene [47]. Our present finding that HNF-6 also synergizes with a more ubiquitously expressed member of yet another family of transcription factors extends the role of HNF-6 in transcriptional regulation.

In conclusion, our data unambiguously show that HNF-6 transactivates the *afp* enhancer by binding to evolutionary conserved sites crucial to its function, both *in vivo* and *ex vivo*. This transactivation by HNF-6 occurs in synergy with the nuclear receptor RORα, thus strongly suggesting that HNF-6 participates in the control of *afp* gene expression. Our results also open the way for more general studies aimed at better understanding the strategies used by HNF-6 to fulfil its function in liver gene expression.

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