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Hierarchy and positive/negative interplays of the hepatocyte nuclear factors HNF-1, -3 and -4 in the liver-specific enhancer for the human α-1-microglobulin/bikunin precursor

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ABSTRACT

Alpha-1-microglobulin and bikunin are two plasma glycoproteins encoded by an α-1-microglobulin/bikunin precursor (AMBP) gene. The strict liver-specific expression of the AMBP gene is controlled by a potent enhancer made of six clustered boxes numbered 1–6 that have been reported to be proven or potential binding sites for the hepatocyte-enriched nuclear factors HNF-1, -4, -3, -1, -3, -4, respectively. In the present study, electromobility shift assays of wild-type or mutated probes demonstrated that the boxes 1–5 have a binding capacity for their cognate HNF protein. Box 5 is also a target for another, as yet unidentified, factor. A functional analysis of the wild-type or mutated enhancer, driving its homologous promoter and a reporter CAT gene in the HepG2 hepatoma cell line, demonstrated that all six boxes participate in the enhancer activity, with the primary influence of box 4 (HNF-1) and box 2 (HNF-4). A similar analysis in the HNF-free CHO cell line co-transfected with one or several HNF factors further demonstrated various interplays between boxes: box 3 (HNF-3α and β) has a negative influence over the major HNF-4 box 2 as well as a positive influence over the major HNF-1 box 4.

INTRODUCTION

Alpha-1-microglobulin (A1M) and bikunin are two plasma glycoproteins found in a free state as well as complexed with other polypeptide chains such as the heavy chains of IgAs for A1M or the heavy chains of Inter-α-Inhibitor family for bikunin (1,2). A1M, a member of the superfamily of lipophilic ligand carriers, i.e. the so-called lipocalins (reviewed in 1,3), is thought to play a role as a carrier for porphyrin or retinol (3,4) and may have a central role in the network of immune regulations (1). Bikunin is a serine protease inhibitor of the Kunitz superfamily and is thought to participate in the control of such events as endothelial cell growth (5) or oocyte cumulus expansion and stabilization (6). Given such a functional importance of both A1M and bikunin, as well as the quantitative fluctuations of these molecules in various pathological states (reviewed in 7), we have been interested in elucidating the regulation of their synthesis at the gene level. Despite their lack of any structural or functional relationship, both A1M and bikunin originate from a shared precursor polypeptide (8) designated α-1-microglobulin/bikunin precursor (AMBP) which is cleaved by a furin-like protease to release the two mature molecules. This AMBP polypeptide is encoded by a single copy gene (9). From rodents to primates, the *AMBP* gene is expressed in the liver exclusively (10–12). We have recently cloned and analyzed the 5′ flanking region of the human *AMBP* gene and we have reported that this gene is under the major control of a potent and tissue-specific enhancer spanning over 144 bp (13). This enhancer is made of six clustered boxes, the sequences of which are potential targets for several hepatocyte-enriched, nuclear factors (HNF) designated HNF-1, -3 and -4. These boxes, numbered 1–6, are arranged in the order HNF-1, -4, -3, -1, -3, -4 (5′ to 3′). This enhancer drives the liver-specific expression of the *AMBP* gene and is in a remote location, 2.7 kb away from the gene promoter which displays a weak and ubiquitous activity. Such an arrangement of a distal and tissue-specific enhancer governing the activity of an ubiquitous promoter is quite unusual for a plasma protein gene (13) and such a large number of HNF boxes clustered within the *AMBP* enhancer is a unique observation. Preliminary electromobility shift assays (EMSA) with nuclear extracts from hepatoma cell lines have demonstrated that boxes 4 and 3 are high affinity binding sites for the HNF-1 or HNF-3 proteins, respectively, while box 1 is a low affinity binding site for HNF-1 (13). However, these experiments did not clarify whether the other three boxes are of functional significance. We now report that all six boxes are indeed involved in the enhancer activity. Moreover,

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their relative importance is quite imbalanced, an HNF-1 box and an HNF-4 box being primarily required for a sustained activity of the enhancer. Finally, our results provide evidence of positive and negative interactions between some HNF factors in the *AMBP* enhancer.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA modification enzymes and Klenow polymerase were obtained from Boehringer Mannheim or Amersham. [α^{35}S]dATP (>600 Ci/mmol), [α^{32}P]dCTP (3000 Ci/mmol), [γ^{32}P]ATP (5000 Ci/mmol), [^{3}H]acetyl-CoA (2–4 Ci/mmol) and X-ray films (Hyperfilm-MP) were obtained from Amersham. Agarose was from Pharmacia. Sterile plasticware for tissue cultures was obtained from Falcon. Minimal essential medium was purchased from Gibco-BRL. Fetal calf serum and antibiotics were from Boehringer Mannheim. Culture grade chemicals used for eukaryotic cell transfections were obtained from Sigma.

Oligonucleotides

Single-stranded oligonucleotides used for sequencing and double-stranded oligonucleotides used in band-shift experiments were purchased from Eurogentech (Belgium) or synthesized onto a Millipore apparatus. Some of these have been previously detailed (13) and include proven targets for various nuclear factors: oligo AlbCCAAT for C/EBP; oligo PE56 for HNF-1; oligo TATHNF3 for HNF-3; oligo PKHNF4 for HNF-4. Some of these oligonucleotides were kindly provided by T. Chouard or B. David-Wattine (Pasteur Institute, Paris) or T. Grange (University Paris VII). Six pairs of sense oligonucleotides numbered with odd numbers 1–11 and their respective, complementary oligonucleotides numbered 2–12, covering the six boxes 1–6 in the *AMBP* enhancer, have also been detailed (13). A series of six sense or antisense oligonucleotides used for site-directed mutagenesis of either box 1–6 in the *AMBP* enhancer were designed to provide a *Xho*I site (CTCGAG) within the mutagenized box(es). They are designated here below by M followed with the box number (the *Xho*I site is underlined):

M1,5′-CCAGCTCGAGTTTTAAAAACAGTCAAAAG-3′ (sense);
M2,5′-AGTCCAAGTGGCCTCGAGCAGCATTTACTC-3′ (sense);
M3,5′-TTTACTCTCTCTCTCGAGTCTGGTTAATAA-3′ (sense);
M4,5′-CTGTTTGCTCTGCTCGAGAATCTCAGGAGC-3′ (sense);
M5,5′-CCTCCAGGAATCTCGAGGCTCCTGAGATTA-3′ (anti-sense);
M6,5′-GGGAGAGGCCCAGAGGATACTCGAGGGATGT TGATTTCT-3′ (anti-sense).

Site-directed mutagenesis of boxes 1–6 in the enhancer

Site-directed mutagenesis at either end of the enhancer (box 1 or 6) was carried out by a single polymerase chain reaction (PCR) using a mutagenizing primer (see above: M1 or M6) and a wild type primer. The wild-type enhancer cloned into a plasmid (see below: constructs) was used as the template. Site-directed mutagenesis of boxes 2–5, which are within the *AMBP* enhancer, was done with two successive rounds of asymetric and symetric PCR (14) using one mutagenizing oligonucleotide (see above: M2-M5) and two flanking wild-type primers. A series of double-mutants, containing two mutated boxes in the enhancer, were obtained by two successive rounds of PCR-aided mutagenesis. In this case, the enhancer with one mutated box was first subcloned into a plasmid and next used as a PCR template for a second mutagenesis step. The GeneAmp 10× PCR buffer and *Taq* polymerase were from from Perkin-Elmer Cetus and the PCR reactions were carried out in a Cetus thermocycler 480. All final PCR products, i.e. the *AMBP* enhancer with one or two mutated boxes, were subcloned into a plasmid with a reporter gene for functional analysis as detailed below. Prior to use, all final constructs were verified by sequencing.

DNA sequencing

Dideoxy-sequencing reactions were carried out with double-stranded, NaOH-denatured plasmid DNA, [α-^{35}S]dATP, and a T7 polymerase sequencing kit (Amersham). The sequencing reactions were separated by electrophoresis onto buffer gradient gels.

Plasmids and constructs

pUC18, -19 and -BM21 were obtained from Boehringer Mannheim. pCH110 (Pharmacia) contains the β-galactosidase gene under the control of the SV40 early promoter. pTKCAT has the chloramphenicol acetyltransferase (CAT) gene under the control of Herpes simplex virus (HSV) thymidine kinase (TK) promoter (–109 to +51). pTK50 is a CAT plasmid with a minimal, 50 bp long HSV TK promoter including an SP-1 box, a TATA box, and a *SalI*/*Hin*dIII/*Xba*I multiple cloning site at the 5′ end of the TK promoter (15). pEMBLCAT6 is a low-background, promoterless CAT plasmid with multiple cloning sites at both ends of the CAT gene (16). A construct containing the minimal *AMBP* promoter (–345 to +57) added with *Sma*I linkers and subcloned at the *Xho*I site (filled-in with Klenow) upstream from CAT in this pEMBL-CAT6 plasmid, is designated pAMBP. Various expression plasmids for the HNF factors included: pRSV-HNF1 (HNF-1α cDNA controlled by Rous sarcoma virus LTR) kindly provided by M. Yaniv; pRB-HNF3A (HNF-3α cDNA controlled by RSV LTR), a gift from K. Zaret; pLEN4S (HNF-4 cDNA controlled by a metallothionein promoter/SV40 enhancer combination), a gift from J. E. Darnell, Jr. The control plasmid pSP(RSV)NN (a gift from T. Williams) is a pSP72-based construct containing the RSV LTR but devoid of any cDNA; it was used for mock-transfections. Likewise, the control plasmid pMTSV40 devoid of the HNF-4 cDNA sequence was prepared in our laboratory from pLEN4S by cDNA excision and plasmid religation.

A series of constructs were made with a wild-type or mutated *AMBP* enhancer (154 bp, –2806 to –2653). The wild-type or mutated *AMBP* enhancer was obtained by PCR with the various oligonucleotides 1–12 and M1–M6 (see above) and was subcloned at the unique *Hin*dIII site (filled-in with Klenow) upstream from the minimal TK promoter in the CAT plasmid pTK50. Finally, from this series of constructs in the p154/TK50 plasmid, the wild-type or mutated *AMBP* enhancer was excised by digestion at the *Sal*I and *Xba*I sites in the pTK50 polylinker and subcloned in anti-sense orientation in pAMBP (see above) at the *Sal*I and *Xba*I sites upstream from the minimal *AMBP* promoter. pW154/AMBP is the resulting construct with the wild-type enhancer, whereas the constructs with one or two boxes mutated within the enhancer are designated with M and the mutated box number(s) in parentheses: p(M1)154/AMBP to p(M6)154/AMBP or p(M1+4)154/AMBP to p(M2+6)154/AMBP are two series of constructs with one or two mutated boxes, respectively.

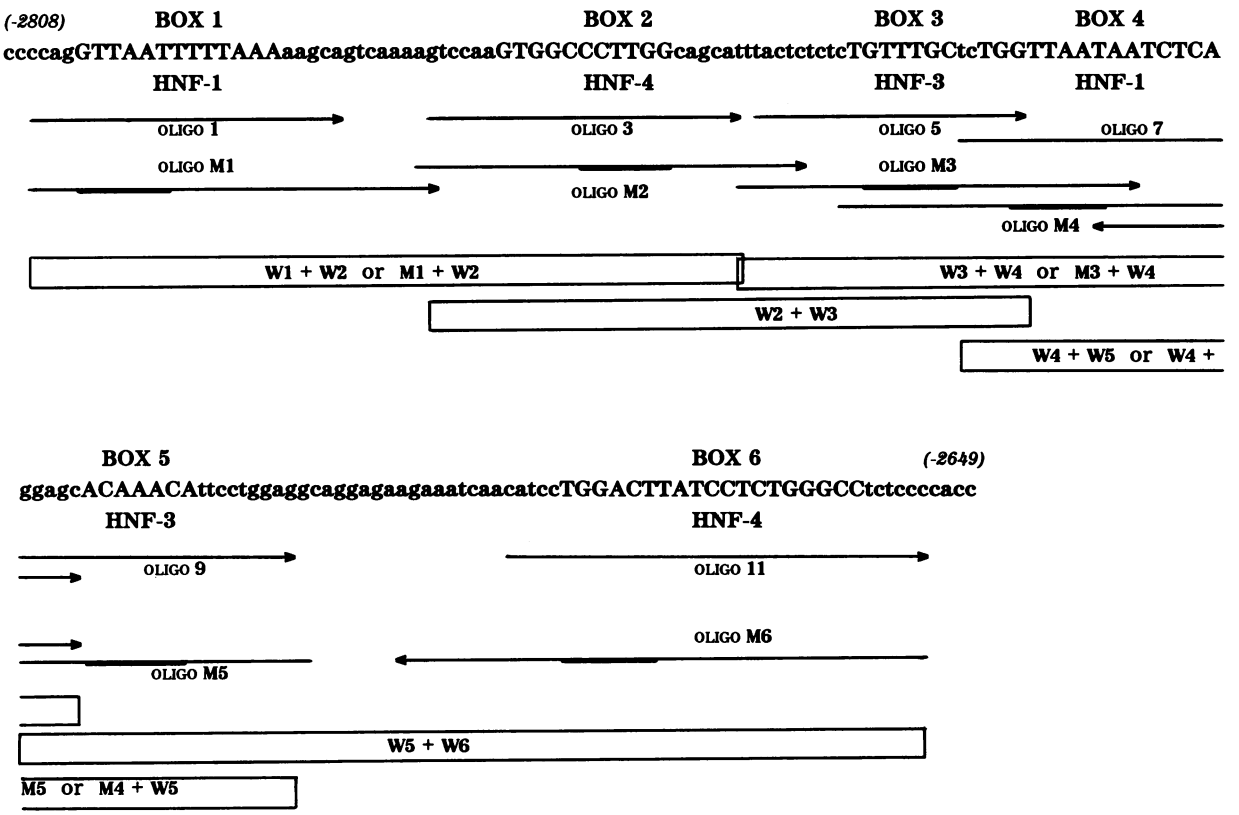


Figure 1. Sequence, arrangement and ligands of the six HNF boxes within the *AMBP* enhancer. The nucleotide sequence of the coding strand in the *AMBP* enhancer is numbered at both ends as in (13). The sequences of the six HNF boxes 1–6 are written with capital letters. Boxes 1, 3 and 4 have a proven binding capacity for their cognate HNF nuclear factor, which is indicated below the line. The potential HNF ligand indicated for the other three boxes 2, 5 and 6 is inferred from sequence alignments with various consensus for HNF boxes (13). Single-stranded, sense oligonucleotides with the wild-type *AMBP* sequence, numbered 1–11 and used for PCR or EMSA (see text), are indicated. Their respective complementary oligonucleotides (numbered 2–12, see text) are not shown. Sense or anti-sense oligonucleotides with a mutated sequence within boxes 1–6 are numbered M1–M6, and the location of the mutated sequence (containing a *Xho*I site, see Methods) is indicated with a thick line. The double-stranded oligonucleotides covering two contiguous HNF boxes and used in EMSA are depicted with an open bar and named from the combination of boxes which they contain, using a 'W' or 'M' prior to the box number when the box is wild-type or mutated, respectively.

Mammalian cell cultures, plasmid transfections and reporter gene assays

The culture of established eukaryotic cell lines including the human hepatoma HepG2 cell line and the chinese hamster ovary (CHO) cell line, as well as the calcium phosphate precipitation procedure used for DNA transfection have been previously detailed (13). In all comparative experiments using the *CAT* gene as a reporter for promoter/enhancer activities, a β-galactosidase reporter plasmid (pCH110) was co-transfected with the CAT plasmid for normalization of CAT activities between culture dishes. A rapid procedure for the simultaneous measurements of β-galactosidase and CAT activities in cell extracts has been reported elsewhere (17). The values of CAT activities were finally expressed as: counts/min [^{3}H]acetyl-chloramphenicol/β-galactosidase unit (13,17).

In transactivation experiments in the CHO cell line, the [*AMBP* enhancer/promoter/CAT] constructs (4 μg) and pCH110 plasmid (1 μg) were co-transfected with one or more expression plasmid(s) for the HNF proteins (listed above) including plasmids for HNF-1α (0.2 μg), HNF-3α (0.2 μg) or HNF-4 (1.0 μg). These amounts of HNF-expressing plasmids were the minimum required for a maximum transactivation by each HNF plasmid used alone, as determined in preliminary experiments. The amount of RSV LTR and/or SV40 enhancer were kept constant in all transfections (mock transactivations versus actual ones) using adequate amounts of the pSP(RSV)NN and/or pMTSV40 plasmid, respectively.

EMSAs

A series of double-stranded oligonucleotides covering a single box 1–6 of the *AMBP* enhancer were obtained by the annealing of two single-stranded oligonucleotides among those listed above (1–12). Likewise, double-stranded oligonucleotides covering two neighbouring, wild-type or mutated boxes within the *AMBP* enhancer (see Fig. 1) were: (i) obtained by PCR with oligonucleotide pairs chosen from those listed above (1–12 and M1–M6) and the wild-type or mutated enhancer subcloned into a plasmid as a template, or (ii) purchased from Genosys (UK) in the case of the W3+W4, W4+W5 and M4+W5 oligos. The double-stranded oligonucleotides used as a probe were end-labeled with [γ^{32}P]ATP and kinase and used for EMSA, as described (13). The nuclear extracts were prepared as in (13). Supershifts of the probes were obtained by addition of 0.5–1 μl of a rabbit anti-HNF antiserum (a kind gift from J. E. Darnell, Jr) to the pre-incubated mixture of labeled probe and nuclear extract, and a further incubation for 15–30 min on ice before loading on gel.

RESULTS

The five boxes 1–5 bind their cognate HNF factor in EMSA

The sequence of the *AMBP* enhancer, along with the arrangement of the six boxes 1–6 in it and the identity of the HNF factor which is a proven or potential ligand for a given box, has been previously published (13). These data are recalled in Figure 1. The recognition of some boxes by HNF proteins were proven by EMSA with nuclear extracts from the human HepG2 hepatocarcinoma cell line and a series of double-stranded oligonucleotides, each covering only one out of the six boxes in the *AMBP* enhancer. We clearly demonstrated that the boxes 1 (HNF-1 binding site), 3 (HNF-3 binding site) and 4 (HNF-1 binding site) are high affinity binding sites for their cognate factor. In contrast, we failed to demonstrate a binding capacity of the isolated boxes 2, 5 and 6 (13). This lack of HNF binding capacity could have resulted from: (i) a spurious identification of potential HNF targets by computer (13), (ii) a too limited number of nucleotides surrounding the HNF target in the probes used (13), or (iii) a requirement for a cooperation with a neighbouring HNF box. To address this issue, double-stranded oligonucleotides covering two contiguous boxes have now been synthesized by PCR (Fig. 1, open bars) and examined for their binding capacity in EMSA. Mutagenesis in a box was further used to confirm the binding capacity of the wild-type sequence.

The binding capacity of AMBP box 2 for the HNF-4 protein is presented in Figure 2. First, a short PKHNF4 probe with a proven binding capacity for the HNF-4 protein (18) contained in a HepG2 nuclear extract was used as a reference (lanes 1–5). The corresponding [HNF-4/PKHNF4 oligo] band was identified by specific (PKHNF4) or non-specific (AlbCCAAT) competitors as well as a super-shift with an anti-HNF-4 antiserum. An AMBP probe covering the single box 1 (probe W1, lanes 6–8) which has a proven HNF-1-binding capacity (13) was our reference for the migration of a slow [HNF-1/HNF-1 oligo] complex which was competed out by a proven HNF-1 site designated PE56 (19). An AMBP probe covering the single box 2 (probe W2, lanes 9–10) incubated with an HepG2 nuclear extract displayed a single band (star in Fig. 2) that corresponds to a non-specific binding (13) but this probe failed to bind HNF-4. A probe W2 with an extended 3′ side (probe W2+W3 in Fig. 1) also failed to bind HNF-4 (data not shown). In contrast, a probe covering both boxes 1 and 2 (probe W1+W2, lanes 11–15) bound the HNF-1 protein in a complex with a slow migration and it also induced the appearance of a faster band clearly identified as a [HNF-4/W1+W2 oligo] complex: a weakening in band intensity was induced by limited amounts of an HNF-4 competitor (PKHNF4, lane 14) and a super-shift of this band was obtained with an anti-HNF-4 antiserum (band SS in lane 15). A probe covering a mutated, non-functional box 1 and a wild-type box 2 (probe M1+W2, lanes 16–18) could no longer bind HNF-1 as judged from the disappearance of the slow, HNF-1-specific band; however, this mutant probe could still bind HNF-4, which was super-shifted with the anti-HNF-4 antiserum (band SS in lane 18). Therefore, the AMBP box 2 can bind its cognate HNF-4 protein as long as a stretch of DNA is present on the 5′ side of this box. However, this requirement does not imply a need for a functional HNF-1 box 1. Finally, the HNF-4 binding capacity of probes W1+W2 and M1+W2 further indicates that a functional box 2 does not require an extended 3′ side.

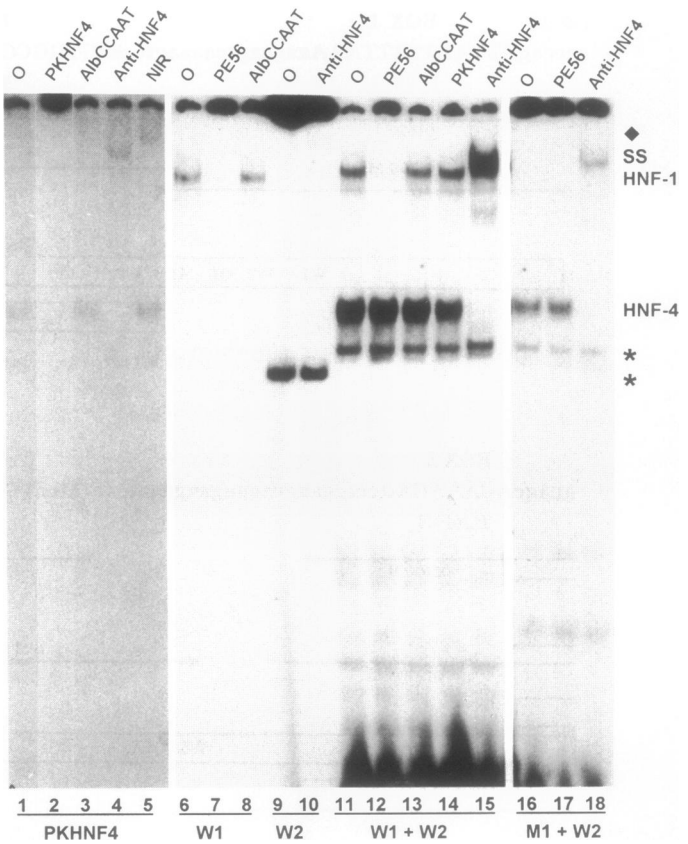


Figure 2. Binding activity of AMBP box 2 (HNF-4) for HepG2 nuclear proteins, studied by EMSA. The labeled, double-stranded oligonucleotide probes used for nuclear factor binding are indicated below the lanes and detailed in Figure 1. Wild-type or mutant AMBP boxes are designated by W or M, respectively, followed with the box number. The competitor oligonucleotides (PKHNF4, 20 ng/lane; all others, 100 ng; O, no competitor) or rabbit antisera (NIR: non immunized rabbit) are listed above the lanes. Anode at the bottom. The migration of major [nuclear protein/oligonucleotide] complexes are identified with the name of the protein. The star denotes a non-specific band associated with box W2 (13). Note that, regardless of their anti-HNF-4 specificity, the rabbit sera used induced the appearance of a faint, non-specific band of very limited mobility (identified with a closed diamond; see lanes 5 and 10); this band cannot be confused with the super-shifted (SS) HNF-4 band (compare lanes 4, 5 and 10).

The binding capacity of AMBP boxes 3 or 5 which harbor a high affinity (box 3) or potential (box 5) binding site for the HNF-3 proteins present in a HepG2 nuclear extract (13) is shown in Figure 3. First, the isolated, wild-type AMBP box 3 was used as a control probe (W3, lanes 7–13). This allowed us to observe a major [HNF-3/W3 oligo] complex seen as a doublet as previously described (13). Assembling this box 3 (HNF-3) together with box 4 (a proven HNF-1 binding site) within a W3+W4 probe (lanes 1–6) resulted in the appearance of this HNF-3 doublet along with an HNF-1 complex observed as a doublet of very slow migration. As expected, this W3+W4 oligo efficiently competed for HNF-3 (lane 10) or HNF-1 (lane 16) bindings. Furthermore, various anti-HNF-3 antisera (lanes 3–6) identified the HNF-3 doublet (lanes 1 and 7) as made of [HNF-3α/box 3] and [HNF-3β/box 3] complexes as we previously suggested (13). When using a probe made of the mutated box 3 and the wild-type box 4 (M3+W4, lanes 14–19), the HNF-3

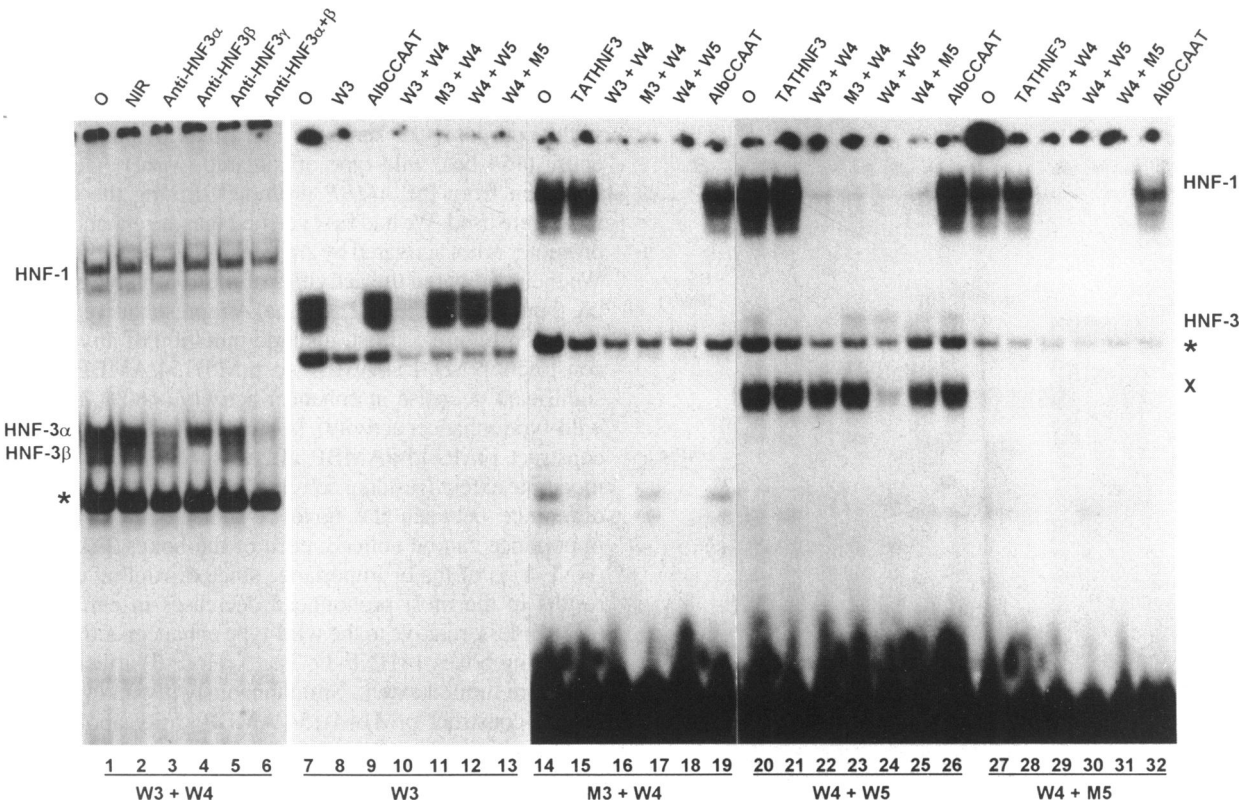


Figure 3. Binding activity of AMBP box 3 (HNF-3) and box 5 (HNF-3) for HepG2 nuclear proteins, studied by EMSA. Lanes 1–6: super-shift experiments; lanes 7–32: competition experiments. The electrophoretic migrations in lanes 1–6 versus 7–32 cannot be compared. Nomenclatures and details as in Figure 2. The star denotes a non-specific band associated with boxes 3 and 5 (13). X denotes a specific complex of box 5 with a protein designated X (see text). Note that band disappearances instead of super-shifts were obtained when using anti-HNF3 antisera (lanes 3–6).

binding capacity of box 3 was abolished whereas the HNF-1 binding capacity of box 4 was retained. Accordingly, this M3+W4 oligo did not compete for HNF-3 binding (lane 11) while it was still able to compete for HNF-1 binding (lane 17).

The putative HNF-3 binding site of box 5 was studied in the context of a probe made of wild-type boxes 4 and 5 (W4+W5, lanes 20–26). This probe displayed an expected HNF-1 binding capacity harbored by box 4 (lanes 20, 21 and 26). It also displayed a very weak binding activity for a protein with an HNF-3 mobility (lanes 20 and 23–26) which was specifically displaced with a HNF3 competitor (TATHNF3, lane 21; W3+W4, lane 22). Such a limited binding capacity of box 5 for HNF-3 was also indicated by the lack of competition of the probe W4+W5 for the association of the [HNF-3/W3 oligo] complex (lane 12). Finally, apart from its HNF-1 binding activity, the major complex formed with the W4+W5 probe and HepG2 nuclear proteins was seen as a fast band (designated 'X' in Fig. 3 and here below) which migrated ahead of all other bands (lanes 20–26). This fast band is clearly associated with box 5 in the probe since mutating the box 5 while retaining the wild-type box 4 (probe W4+M5, lanes 27–32) abolishes the occurrence of this band and prevents the corresponding oligonucleotide W4+M5 to compete for the formation of this fast band (compare lanes 24 and 25). The protein X involved in this band was not further identified in this study. However, competition experiments with oligonucleotides covering proven binding sites for factors of the HNF-1- (lanes 22–23), HNF-3- (lanes 21–22), or C/EBP (lane 26) families as well as the lack of reactivity of the protein X with anti-HNF-3 or anti-HNF-4 antisera (see below Fig. 4, lanes 11–14) make a relationship of the protein X with either of these families unlikely. We also observed that this protein X, found in HepG2 hepatoma cells, is detected in a HeLa nuclear extract as well (result not shown). In conclusion, our results indicate that AMBP box 5 has a very weak binding capacity for HNF-3 and a major binding capacity for another factor designated X. Although they were not mentioned in our previous report (13), these binding activities of box 5 for HNF-3 and X are visible when using a short probe made of the box 5 alone and an extended autoradiographic exposure (not shown). Therefore, these binding activities do not depend on the nucleotide environment and/or nuclear proteins on the 5′ or 3′ side of box 5. In keeping with this conclusion, an M4+W5 probe still allowed for binding of HNF-3 and X onto box 5 while the HNF-1 binding capacity of box 4 was lost (result not shown).

The binding capacity of AMBP box 6, that has been described as a potential HNF-4 binding site (13), was investigated with a W5+W6 probe, as shown in Figure 4. A W1+W2 probe (i.e. HNF-1 + HNF-4 sites) was used as a control for migration of a [HNF-4/probe] complex (lanes 1–3 and 7–9). With the W5+W6 probe and an HepG2 nuclear extract two major bands were obtained (Fig. 4, lanes 4–6 and 10–15; see star and X). They correspond to a non-specific band (star) and the [protein X/probe] complex that are associated with box 5 (see Fig. 3, W4+W5). Indeed, competition experiments with HNF-3 or -4 oligos did not displace either of these bands (Fig. 4A, lanes 5–6). Super-shift

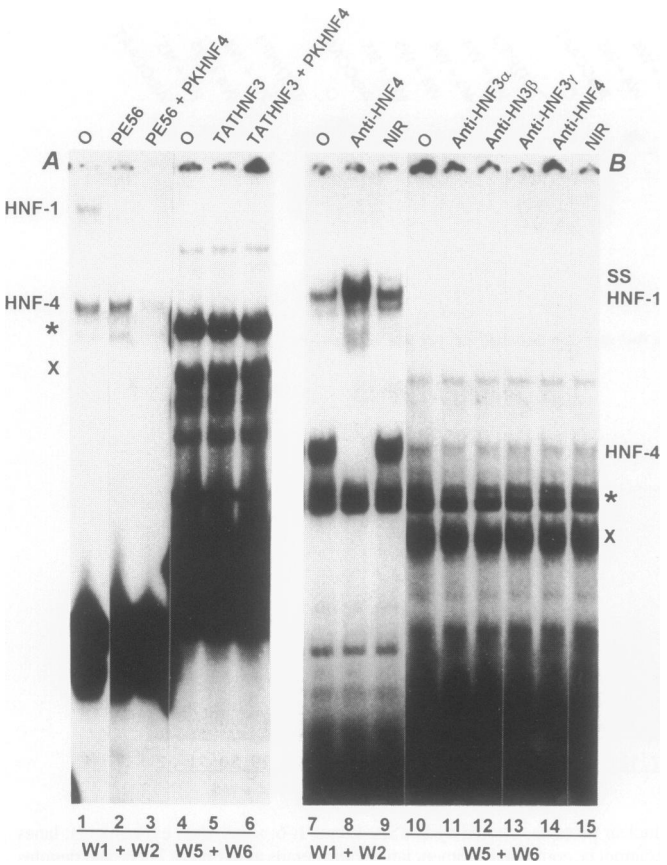


Figure 4. Binding activity of AMBP box 6 (HNF-4) for HepG2 nuclear proteins, studied by EMSA. (**A**) Competition experiments. (**B**) Super-shift experiments. Nomenclatures and details as in Figure 2. The star denotes a non-specific band associated with box 5 (13) and X denotes a specific complex of box 5 with an uncharacterized protein designated X and first identified in Figure 3.

experiments with anti-HNF-3α, -β, or -γ or anti-HNF-4 antisera (Fig. 4B, lanes 11–14) failed to shift any of the bands formed with this W5+W6 probe. These data indicate a lack of HNF-4-binding capacity for the box 6. In fact, from our EMSA experiments, no binding activity whatsoever could be attributed to box 6.

In conclusion, this part of our study allowed us to observe that boxes 1–5 in the *AMBP* enhancer are able to bind their cognate HNF factor as well as an unidentified factor X on box 5. A binding activity for box 6 was undetectable. Most of these boxes display a binding capacity even though they lack their natural nucleotide environment as shown for boxes 1, 3, 4 (13) and 5 (this study). When an extended stretch of DNA next to a given box is required for the binding capacity of this box (box 2), this stretch does not need to be a functional target for an HNF protein. In general, ablating the binding capacity of either of boxes 1–5 does not enhance or abolish the binding capacity of a neighbouring box as judged from mutated probes covering the box pairs 1+2, 3+4 or 4+5. Therefore, the binding of a nuclear factor on either of these boxes does not seem to require a cooperation with a [box/factor] combination located next to it.

In hepatoma cells, the boxes 1–6 within the whole enhancer are functional with a hierarchy

Our EMSAs demonstrated that replacing any box 1–5 by a *Xho*I sequence fully abolished the HNF-binding capacity of such mutated boxes. Therefore, with the same *Xho*I mutational approach we next examined which boxes are functional in the context of the whole *AMBP* enhancer and the HepG2 hepatoma cell line that synthesizes all three HNF-1, -3 and -4 proteins. For such a purpose, the series of constructs p154/AMBP with the entire (154 bp), wild-type or mutated (*Xho*I) enhancer located upstream from the *AMBP* promoter driving the CAT reporter gene were used. We had first verified that the enhancerless *AMBP* promoter is not activated by any of the HNF factors (not detailed). We then compared the activities of these constructs by transfecting them into the HepG2 cell line. A representative experiment is shown in Figure 5. First, a single mutation of any box 1–5 [i.e. constructs p(M1)154/AMBP to p(M5)154/AMBP] results in a significant decrease in enhancer activity (<60%, relative to the wild-type enhancer activity). In contrast, mutating the box 6 in the construct p(M6)154/AMBP affects the enhancer activity to a moderate extent (residual activity >80%). Furthermore, a marked difference between the boxes 1–5 in terms of their relative importance can be noticed: each of the boxes 2 (HNF-4) and 4 (HNF-1) is of major importance since disrupting either of them results in the most pronounced decreases in enhancer activity (15% or less, relative to the wild-type enhancer activity), whereas disrupting boxes 1 (HNF-1), 3 or 5 (HNF-3) affects this activity to a more limited extent. Simultaneously disrupting both HNF-1 boxes [construct p(M1+4)154/AMBP] fully abolishes the enhancer activity as expected from a construct in which box 4 is disrupted. In contrast, simultaneously disrupting both HNF-3 boxes or the HNF-4 box 2 along with the box 6 results in a still limited loss of enhancer activity (residual activity 15–20%, see lowest two lines in Figure 5). Notably, the residual activity of the enhancer doubly mutated at boxes 2 and 6 is similar to what is seen with a single mutation in box 2. This confirms the quite limited involvement of box 6 in the enhancer activity. Finally, the residual activity of the enhancer doubly mutated at the HNF-3 boxes 3 and 5 indicates an additive effect of these boxes upon the enhancer activity. Overall, in the context of the complete *AMBP* enhancer and the HepG2 cells our results indicate a hierarchy in the relative importance of the boxes 1–6 and their cognate HNF proteins as follows: boxes 2 and 4 > boxes 1, 3 and 5 > box 6; and HNF-1 > HNF-4 > HNF-3. These observations and conclusions were also obtained in further studies where the *AMBP* enhancer was studied in the context of a foreign promoter, namely the minimal, 50 bp long HSV TK promoter (results not detailed).

Functionality of the HNF boxes within the whole enhancer transactivated with a limited number of HNF factors

We wished to investigate whether the influence of a box and its cognate HNF factor upon the overall activity of the *AMBP* enhancer could depend on the simultaneous presence and activity of other box(es) and HNF factor(s). Therefore, we have investigated the activity of the full-length enhancer when one or more HNF factor(s) are provided in the non-hepatocytic CHO cell line by co-transfecting expression plasmids for the HNF proteins. Preliminary experiments with the pAMBP plasmid containing the minimal, enhancerless *AMBP* promoter (–345 to +57) upstream from the *CAT* gene indicated that this construct did not respond to a transactivation in CHO cells co-transfected with an expression plasmid for any HNF factor (results not shown). On these grounds, our study was performed with the series of

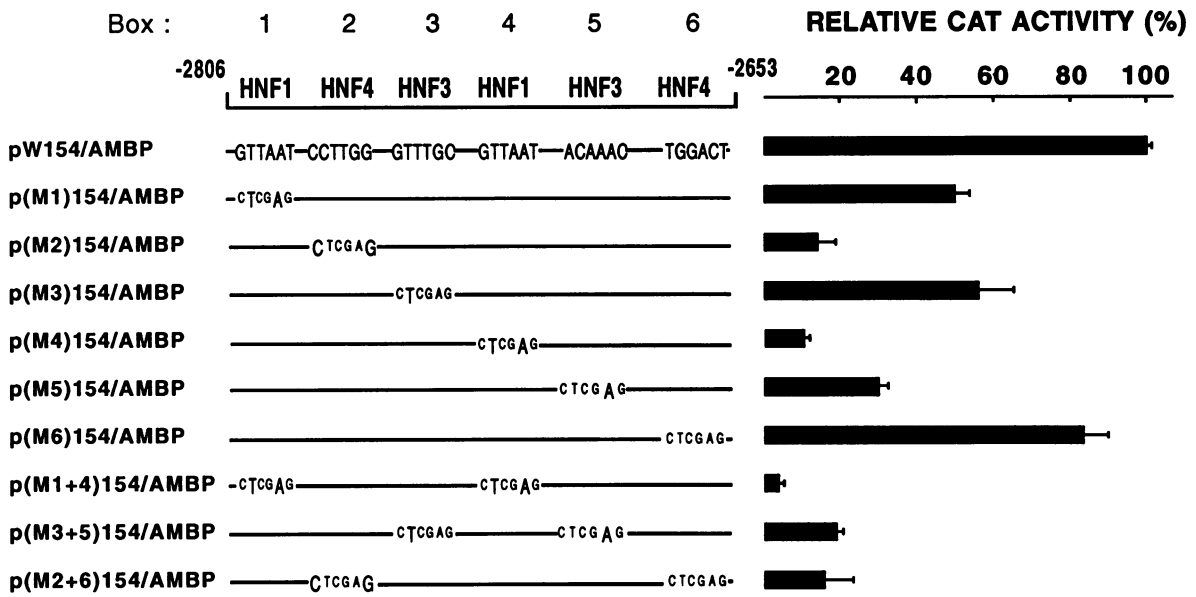


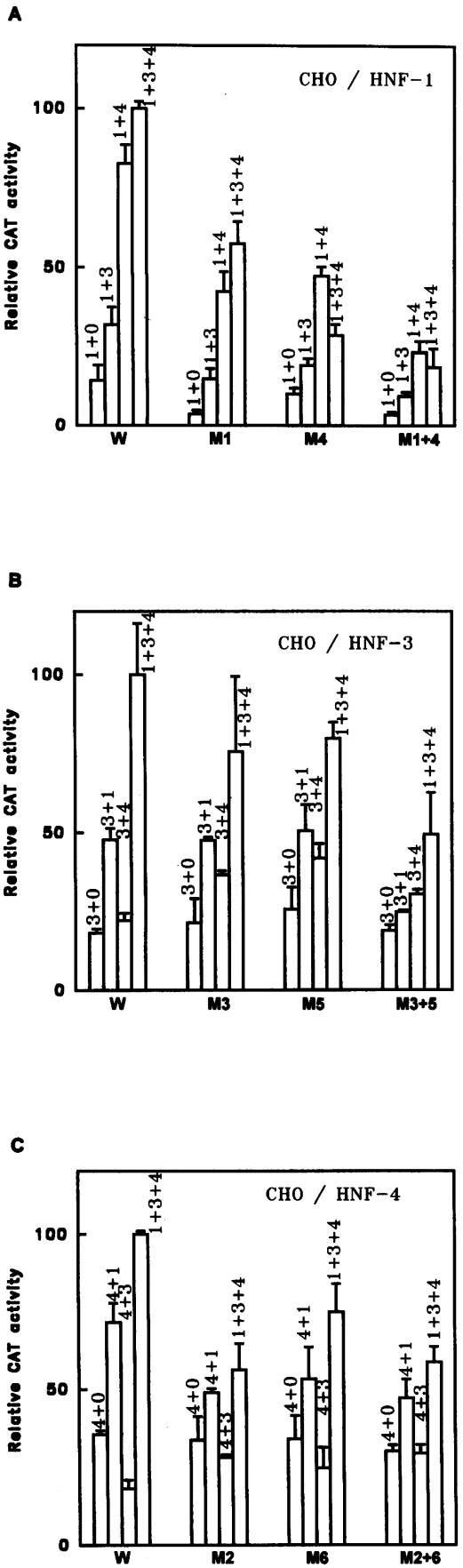
Figure 5. Activity of CAT constructs with a minimal *AMBP* promoter under control of the wild-type or mutated *AMBP* enhancer, in the HepG2 hepatoma cell line. Left column: List of CAT plasmids with the wild-type *AMBP* enhancer (pW154/AMBP, top line) or the enhancer mutated within one box (M1–M6 series, central six lines) or two boxes (M1+4 to M2+6 series, last three lines). Central panel: Details of wild-type or mutated sequences within the enhancer boxes 1–6. The boxes 1–6 with their cognate HNF factor are recalled at the top (the distances between boxes are not to scale). The wild-type sequence is on the first line, with the six target sequences for site-directed mutagenesis written in capitals. The mutated sequence within each box (lowest 9 lines) results in a *Xho*I site (CTCGAG) where the nucleotides that depart from the wild-type sequence are written in lower case letters while those which respect the wild-type sequence are in capitals. Right panel: Relative CAT activities (% of maximum) of the CAT constructs are shown as mean ± S.D. (n = 3).

constructs containing the normal or mutated *AMBP* enhancer driving its homologous promoter and the CAT gene in pAMBP, i.e. the series of constructs pW154/AMBP to p(M2+6)154/AMBP. Repeated experiments provided the representative results shown in Figure 6.

The wild-type or mutated AMBP enhancer (noted W or M in Fig. 6) was tested in the presence of HNF-1α (Fig. 6A), HNF-3α (Fig. 6B) or HNF-4 (Fig. 6C) expressed alone or in combinations. The CAT activities can be compared from Figure 6A–C as they are relative to that of the wild-type enhancer in pW154/AMBP set as 100% when transactivated by all three HNF-1, -3 and -4 (this combination of factors is noted 1+3+4 in Fig. 6). The data in Figure 6 confirm our conclusions above that: (i) a disruption of any box 1–6 affects the enhancer activity (in A–C, with 1+3+4: compare M versus W), and (ii) the boxes 2 (HNF-4) and 4 (HNF-1) are of primary importance for this activity (A–C, with 1+3+4: M2 and M4 versus others).

Further conclusions were drawn from these transactivation experiments. The HNF-1 protein alone is able to promote an enhancer activity since disrupting either HNF-1 target, i.e. box 1 or 4, results in a drop in this HNF-1-induced activity of the enhancer (+0 in A: M versus W). In contrast, the importance of the other four boxes is not seen when their cognate protein is expressed alone (+0 in B or C: M versus W). However, the activity of these boxes is observed when given combinations of HNF proteins are present. In particular, a complex influence of HNF-3 is seen, as follows. First, when the HNF-3 and HNF-4 proteins are simultaneously present (3+4), a transactivating capacity of HNF-4 is not observed with the wild-type enhancer (B, W: 3+0 versus 3+4) whereas it is seen if at least one HNF-3 box (box 3 or 5) is disrupted (B, M: 3+0 versus 3+4). This indicates an hindrance in transactivation resulting from the simultaneous presence of both HNF-3 and HNF-4 proteins onto the wild-type enhancer. This is fully confirmed by the negative effect of HNF-3 upon the HNF-4-induced activity of the wild-type enhancer (C, W: 4+0 versus 4+3). This negative effect is also seen with the enhancer deprived of box 6 (C, M6: 4+0 versus 4+3) but it is no longer seen when box 2 is disrupted (C, M2: 4+0 versus 4+3; and M2+6: 4+0 versus 4+3). This implies that the main target of a negative interference between HNF-4 and HNF-3 is box 2.

Secondly, when the HNF-3 and HNF-1 proteins are simultaneously present (3+1), disrupting either of the HNF-3 boxes 3 or 5 does not affect the transactivation of the enhancer by HNF-1 (B, 3+1: W versus M3 or M5) whereas disrupting both HNF-3 boxes 3 and 5 abrogates this *trans*-activation by HNF-1 (B, 3+1: W versus M3+5). This indicates that a cooperation of HNF-3 and HNF-1 is involved in the HNF-1-induced activity of the enhancer and requires at least one HNF-1 and one HNF-3 box. Further data in Figure 6 indicate that the HNF-1 box 4 is the target of such a cooperation, as follows. With the simultaneous presence of all three HNF proteins the net effect of HNF-3 upon the wild-type enhancer is a positive one since (i) adding HNF-3 to HNF-1 and HNF-4 increases the enhancer activity (W in A and C: 1+4 versus 1+3+4) and (ii) a limited drop (30–50%) in the enhancer activity is observed when disrupting one or both HNF-3 boxes (B, 1+3+4: M versus W). Likewise, the enhancer deprived of box 1 is positively regulated by this set of three HNF factors (A, M1: 1+4 versus 1+3+4). In contrast, the enhancer deprived of box 4 is negatively regulated by the same set of 3 HNF factors (A, M4: 1+4 versus 1+3+4). This indicates that in the enhancer deprived of box 4 a positive effect of HNF-3 upon the HNF-1 box 4 is lost



while the remaining negative effect of HNF-3 upon the HNF-4 box 2, as described above, results in a negative net effect of the 3 HNF proteins.

DISCUSSION

The nuclear factors that are mostly responsible for the tissue-specific transcription in fully differentiated liver cells include HNF-1α, a member of the *POU-homeobox* gene family, HNF-3α, -β and -γ that share homologies with the *Drosophila* homeotic gene *fork head*, HNF-4, an orphan member of the steroid hormone receptor superfamily, and C/EBPα, -β and DBP which are members of the leucine zipper dimerization family (20–23). The transcription elements that control the strictly liver-restricted expression of the *AMBP* gene are comprised of a strong and distal enhancer and a weak and ubiquitous promoter. This unusual arrangement together with the unique clustering of six potential boxes for the HNF-1, -3 and -4 factors within the *AMBP* enhancer make the latter a powerful and exciting model for investigating the role of various DNA binding proteins in the activity of tissue-specific enhancers. Accordingly, the current study was undertaken for two major purposes. First, we wished to clarify whether all six potential binding sites for HNF proteins within the *AMBP* enhancer are indeed functional. Secondly, interactions between different transcription factors bound to adjacent sites can lead to striking changes in their respective activities and we wished to analyze such a possibility in the context of an unusually high number of tightly clustered HNF boxes.

In our former study (13), boxes 1, 3 and 4 in the *AMBP* enhancer were identified by EMSA as genuine targets for the HNF-1, HNF-3 and HNF-1 proteins, respectively, but the functionality of boxes 2, 5 and 6 remained unproven. The present EMSA experiments were carried out with extended probes containing two wild-type or mutated boxes of the *AMBP* enhancer. This allowed us to unambiguously demonstrate that box 2 binds HNF-4 and box 5 binds HNF-3 as initially predicted from these box sequences. In contrast, we failed to observe any [HNF-4/box 6] complex although box 6 is functional as indicated by mutagenesis experiments of [*AMBP* enhancer/CAT] constructs. That both boxes 2 and 6 are potential targets for HNF-4 was initially based on the different proposals of consensus sequences for HNF-4 (13). More recent proposals have distinguished a consensus for the HNF-4 factor (24) from another consensus for both HNF-4 and COUP-TF factors (25). The box 6 sequence is close to the latter consensus but an analysis of

Figure 6. Activity of CAT constructs with a minimal *AMBP* promoter under control of the wild-type or mutated *AMBP* enhancer in the CHO cell line co-transfected with expression plasmids for the HNF proteins. The CAT constructs were studied in an HNF-1α (**A**) or HNF-3α (**B**) or HNF-4 background (**C**). This background was tested alone (+0) or was supplemented with HNF-1α (+1), HNF-3α (+3) or HNF-4 (+4) as indicated above the bars. The *AMBP* enhancer in the constructs is of the wild type (W) or is mutated in either of boxes 1–6 (M1–M6) or in two boxes simultaneously (M1+4 or M3+5 or M2+6) as indicated beneath the bars. The relative CAT activities (mean ± S.D., n = 3) are expressed as % of a maximum (100%) which is the activity of the wild-type *AMBP* enhancer in the HNF context: +1+3+4. The background activity of the wild-type *AMBP* enhancer in CHO cells deprived of any HNF factor is 8%.

AMBP enhancer/CAT constructs co-transfected with a COUP-TF expression plasmid in the HepG2 cells did not give consistent data (not shown). Therefore, we still do not know whether the box 6 binds a member of the steroid hormone receptor superfamily. Our former (13) and present data have further demonstrated a strong affinity of HNF-1 or HNF-3 for box 4 or 3, respectively, which is to be opposed to the low affinity of HNF-1 or HNF-3 for box 1 or 5, respectively. In fact, given this very low affinity of HNF-3 for box 5 the quantitatively major nuclear protein detected onto box 5 is not HNF-3 but instead another protein designated X. The preliminary data of this study indicate that this protein X is apparently not related to any known family of liver-enriched factors. Its identity is currently investigated.

For any box from 1 to 5, the specific binding of a nuclear protein takes place regardless of the wild-type or mutated sequences surrounding the binding site and therefore this binding does not call for the presence of another factor in a close position. This conclusion is corroborated by the lack of any supershifted band in EMSAs (Figs 2–4 and results not shown) that would have been seen if a cooperative binding of two factors (26) took place onto neighbouring boxes in a single probe molecule. However, we cannot exclude that a cooperativity at the binding step involves some distant [box/factor] complexes in the enhancer. In this respect, footprinting experiments have been performed but, as yet, they have only indicated that a complex array of factors protects the *AMBP* enhancer (data not shown).

The experiments with wild-type or mutagenized [*AMBP* enhancer/CAT] constructs were first carried out in the HepG2 hepatoma cells in which the relative amounts of HNF-1, HNF-3 and HNF-4 proteins are close to those found in the fully differentiated liver. The data thus obtained indicate a hierarchy in the relative importance of boxes 1–6 and their cognate HNF proteins for the overall activity of the whole enhancer, as follows: boxes 2 and 4 > boxes 1, 3 and 5; and HNF-1 > HNF-4 > HNF-3. This HNF hierarchy, however, is valid only for the enhancer under study since it is, at least partly, the reflection of interplays between some boxes as next observed in the CHO cell line. Indeed, adding stepwise either of the HNF factors to the HNF-free CHO cells allowed us to further identify positive or negative interferences between boxes. Specifically, either of the HNF-3 boxes 3 or 5 is sufficient to increase the HNF-1-induced, enhancing activity of box 4, while box 3 (and seemingly box 5) negatively regulates the HNF-4-induced, enhancing activity of box 2. Therefore the complete *AMBP* enhancer, as depicted in Figure 7, first consists of two major boxes, namely box 2 (HNF-4) and box 4 (a high affinity site for HNF-1), that both have a strong enhancing capacity. They are separated by box 3, a high affinity site for HNF-3, that negatively regulates box 2 activity and positively regulates box 4. Likewise, box 5, a very weak binding site for HNF-3 and also a target for the protein X, positively regulates box 4 activity. Our data obtained with the HepG2 and CHO cells show that the net result of the HNF-3-driven positive/negative interplays is a positive one when the HNF-1 protein is present and its cognate boxes are active. Therefore the negative HNF-3/HNF-4 interference observed in the CHO cells is likely to be of limited importance when the wild-type enhancer is in the context of a fully differentiated and quiescent hepatocyte with all three HNF-1, -3 and -4 proteins. Finally, box 1 (a weak affinity site for HNF-1) and box 6 add further strength to this enhancer but are apparently not involved in any partnership with another [box/HNF protein] complex. Such a functional arrangement of six boxes including five proven targets for the three families of HNF factors clustered within a liver-specific enhancer has not been reported before.

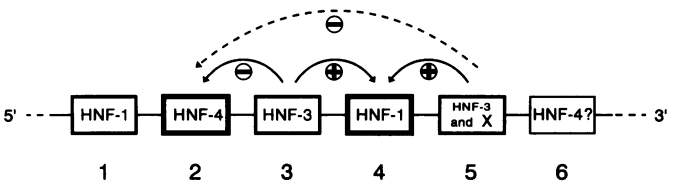


Figure 7. Current view of the *AMBP* enhancer: hierarchy and interplay between [box/factor] units. Each box is depicted with a rectangle and numbered from 1 to 6. The cognate nuclear factor(s) is (are) indicated inside the box. The quantitative participation of a given [box/factor] unit in the overall enhancer activity is grossly proportional to the thickness of the corresponding rectangle. A positive (+) or negative (–) influence of a [box/factor] unit upon another one is indicated with a curved arrow. Given the very low binding capacity of HNF-3 onto box 5 (see text), the negative influence of [box 5/HNF-3] upon box 2 is likely to be quite limited (dotted arrow). The possible interferences of the [box 5/protein X] complex with other boxes are unknown. The box 6 positively participates in the enhancer activity but its cognate nuclear factor (possibly a member of the HNF-4 family?) remains to be identified.

As discussed above, our EMSA results did not indicate any requirement of a [box/HNF factor] unit for the binding of another HNF factor onto a neighbouring box. Therefore, we conclude that interplays between given [box/factor] units in the *AMBP* enhancer take place after the binding steps, i.e. at the activation step. Positive or negative interactions between several [factor/box] units, whether they are located in a close vicinity or at a distance, have been described for several combinations of liver-enriched nuclear factors, such as HNF-1 and HNF-3 (27,28), HNF-1 and HNF-4 (29), HNF-1 and C/EBP (30), HNF-3 and NF1 (31), HNF-4 and C/EBP (32). However, the exact nature of the interactions involved is sometimes unknown or, when it is clarified, it applies only to the particular gene (28) or to the constructs and experimental conditions (31) under study. Indeed, the presence of identical boxes and factors can induce opposite effects in different genes. For instance, the negative HNF-4/HNF-3 interplay seen in the *AMBP* enhancer cannot be compared with the negative HNF-1/HNF-3 interplay seen with the *aldolase B* gene (28) since the latter case results from a competition of factors for overlapping sequences, whereas the boxes 2 and 3 in the *AMBP* enhancer are apart from each other (Fig. 1). In one instance, however, is the *AMBP* enhancer activity reminiscent of what has been previously reported for another gene. Indeed, it has been shown that HNF-3 functions as both a transcriptional activator and an organizer of chromatin structure that establishes hepatocyte-specific accessibility of other factors within specific target genes (27). This might account for the positive HNF-1/HNF-3 interplay seen in this study. Overall, our present data brings further evidence that the same limited set of liver-enriched factors can regulate various genes in a quite different fashion depending on the number, arrangement, and affinity of the corresponding [box/factor] units in these genes.

Finally, our data point to a dual, positive and negative influence of HNF-3 upon the activity of the *AMBP* enhancer that primarily is under the positive control of HNF-4 and HNF-1 (Fig. 7). Therefore, limited changes in the level of either of these nuclear factors (as mimicked in this study with CHO cells) could

participate in the fine tuning of *AMBP* gene transcription that takes place during such events as liver development (11) or an inflammatory acute phase response (7).

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