Members of the CAAT/enhancer-binding protein, hepatocyte nuclear factor-1 and nuclear factor-1 families can differentially modulate the activities of the rat α-fetoprotein promoter and enhancer

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The promoter of the rat α-fetoprotein (AFP) gene, which makes the expression of the developmentally regulated AFP gene specific to the liver, is a putative target for transcription factors of the CAAT/enhancer-binding protein (C/EBP), hepatocyte nuclear factor-1 (HNF-1) and nuclear factor-1 (NF-1) families. We have evaluated the influence of these factors on the activity of the AFP promoter by transfection of HepG2 hepatoma cells with the appropriate expression vector plus a CAT plasmid under the control of the AFP promoter. A similar plasmid bearing the rat albumin promoter was used as a control. C/EBPα, C/EBPβ and D-binding protein (DBP) acted as *trans*-activators on the AFP promoter, whereas liver inhibitory protein (LIP), a truncated form of C/EBPβ, was a potent negative regulator of the promoter. C/EBPα also bound to and stimulated the activity of the AFP enhancer at −2.5 kb. Interestingly, HNF-1β was found to be more potent than HNF-1α in activating the AFP promoter. This effect was specific, as it did not occur with the rat albumin promoter. HNF-1β, which is produced earlier than HNF-1α during liver development, would thus have the greater influence on the AFP promoter in early development. Both HNF-1s allowed expression of the AFP promoter in cells of non-hepatic origin. Overexpression of NF-1 induced a specific decrease in the activity of the AFP promoter. This strongly suggests that competition between NF-1 and HNF-1 for binding to their overlapping binding sites on the AFP promoter is critical for modulating its activity. Thus changing combinations of these *trans*-acting factors may tightly modulate the AFP promoter activity in the course of liver development and carcinogenesis.

INTRODUCTION

Cellular differentiation requires a succession of precise and tightly controlled combinations of several *trans*-acting factors. These combinations initiate and maintain the transcription of a range of genes in a cell-specific manner. A considerable amount of information has been obtained over the past few years about the molecular mechanisms governing the expression of genes in liver cells during their differentiation. Several *trans*-acting factors that could be involved in the transcription of liver-specific genes, such as albumin, α-fetoprotein, α-antitrypsin, fibrinogen, transthyretin and transferrin, have been discovered and characterized. Proteins such as the hepatocyte nuclear factors, HNF-1, HNF-3 and HNF-4, and the CAAT/enhancer-binding proteins (C/EBPs) are representative of liver-enriched *trans*-acting factors that may play key roles in the transcription of genes in the liver cell (see refs. [1–5] for reviews). Their cDNAs have been cloned after purification of the protein or, more directly, by using their binding site on DNA-regulatory elements as a probe to screen an expression library. This approach has led to the discovery of several distinct but related proteins that form families of transcription factors. Some members of these families of transcription factors, which generally bind to DNA as homo- or heterodimers, exhibit a preferential tissue- and developmental-specific pattern of expression. This probably serves as a means of generating diversity in terms of signals allowing modulation of gene expression in the liver cells in response to different stimuli.

The α-fetoprotein (AFP) gene is a good model system with which to learn more about the molecular mechanisms involved in regulating the expression of liver-specific gene expression during development and carcinogenesis. The AFP protein, the precise role of which is not yet fully established, is the major plasma protein during fetal life in mammals. The AFP gene belongs to a family that includes the albumin- and vitamin D-binding protein, and its expression is mainly regulated at the transcriptional level (see refs. [6 and 7] for reviews). Transcription of the AFP gene occurs mainly in the yolk sac, fetal liver and intestine. It falls abruptly around birth and is almost totally blocked during normal adult life [8]. However, it can resume under certain pathophysiological conditions, such as liver regeneration after partial hepatectomy or chemical intoxication or in liver carcinogenesis (see refs. [9 and 10] for reviews).

We and others have used several complementary approaches to show that the promoter region is responsible for the specificity of AFP gene expression in the liver and participates in the down-regulation of the AFP gene transcription induced by glucocorticoids [11–15]. These studies have also clearly demonstrated that there are several regulatory elements in the 5′ extragenic region of the AFP gene in different species. Most of these results are consistent with those from *in vivo* studies performed with transgenic mice [16,17]. These latter studies also indicate that the AFP enhancer elements may remain active during adult life, and that the region between −250 and −838 is probably involved in the postnatal repression of AFP transcription in the liver.

In vitro DNA–protein-binding experiments, mutation analysis and transient-transfection experiments have shown that the liver-restricted transcription factor HNF-1 can bind to two regions of the AFP promoter and may play a major role as a *trans*-activator [3,12,18–20]. C/EBP and the ubiquitous factor, nuclear factor-1 (NF-1), which is involved in both replication and transcription

Abbreviations used: AFP, α-fetoprotein; C/EBP, CAAT/enhancer-binding protein; HNF-1, hepatocyte nuclear factor-1; NF-1, nuclear factor-1; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; LIP, liver inhibitory protein; DBP, D-binding protein.

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processes, also bind to the AFP promoter [7,18,21]. The description of both these *cis*- and *trans*-acting elements was an essential prerequisite for determining how the AFP promoter may function in the liver cell specifically. The next goal now is to determine how individual members of these families of transcription factors and their combination can participate in the modulation of the activity of the AFP promoter itself and of the AFP-regulatory elements during development or carcinogenesis.

The availability of cloned cDNAs for these factors now makes it possible to explore these ways. As a first step, we have evaluated the modulating ability of several transcription factors of the C/EBP, HNF-1 and NF-1 families on the AFP promoter activity by co-transfection and transient-expression experiments. The vectors used allowed expression of these transcription factors and the CAT gene under the control of the AFP promoter in cells of hepatic or non-hepatic origin.

MATERIALS AND METHODS

Materials

Chemicals and reagents used in this study were obtained from the following sources: silica plates from Merck (Chelles, 77502, France), protein assay reagent from Bio-Rad (Ivry sur Seine, 94203, France), acetyl- and butyryl-CoA and 2,6,10,14-tetramethylpentadecane from Sigma (Saint Quentin Fallavier, 38297, France), [^{14}C]chloramphenicol and [γ-^{32}P]ATP from Amersham (Les Ulis, 91944, France), modifying and restriction enzymes from New England Biolabs (Montigny le Bretonneux, 78180, France) and additional chemicals from Merck, Sigma or Aldrich (Saint Quentin Fallavier, 38297, France).

Plasmids

Chloramphenicol acetyltransferase (CAT) vectors

Plasmids pBL-CAT2 and 3 [22] were used as starting material to construct plasmids bearing the CAT gene under the control of rat AFP or albumin gene promoters. The *Sac*I–*Xba*I 1 kb fragment (named UMS) from the upstream region of the mouse c-*mos* gene was cloned just in front of the polylinker region of these plasmids (plasmids pBl-UMS-CAT2 and 3) to prevent the contribution of any spurious initiation of transcription in the plasmid [23]. Conventional cloning procedures were used to insert the −197 to +6 (*Bbv*I–*Msp*I) region of the rat AFP promoter [24] into the *Hin*dIII–*Bgl*II sites of pBL-UMS-CAT3 yielding plasmid pBL-UMS-AFP26-CAT. This plasmid is referred to as pAFP-CAT throughout.

Plasmid pBL-PO31-tk-CAT5 contains the 170 bp *Hin*dIII–*Bgl*II enhancer region at −2.5 kb from the AFP gene [13] cloned in the natural orientation into the blunted *Sal*I site in the polylinker of plasmid pBL-CAT5 [25] in front of the thymidine kinase (tk) promoter of the herpes simplex virus.

Plasmid pBL-UMS-ALB-CAT (pALB-CAT) was obtained by cloning the −175 to +15 (*Alu*I–*Hin*cII) region of the rat albumin promoter [19] instead of the −197 to +6 region of the AFP promoter at the *Hin*dIII–*Bgl*II sites in plasmid pAFP-CAT.

pSV2-CAT plasmid contains both the promoter and the enhancer region of simian virus 40.

Other expression vectors

pMSV-C/EBP [26] was obtained from Dr. S. McKnight (Carnegie Institution of Washington, Baltimore, MD, U.S.A.), pCMV-LAP [27], pCMV-LIP [28], and pCMV-DBP [29] were obtained from Dr. U. Schibler (University of Geneva), pRSV-HNF1 [30] and pRSV-vHNF1 [31] were from Dr. M. Yaniv (Institut Pasteur, Paris, France), and pRSV-NF21 [32] was from Dr. R. Cortese (Instituto di Ricerche di Biologia Moleculare, Roma, Italy). They allow expression of C/EBPα, C/EBPβ, LIP, HNF-1α and -1β and NF-1 in eukaryotic cells.

All the plasmids were purified by two successive ultracentrifugation steps through caesium chloride gradients.

Preparation and partial purification of recombinant C/EBPβ protein

Bacteria carrying the C/EBPβ cDNA under the control of a T7 polymerase-dependent promoter [27] were obtained from Dr. Schibler. They were grown in a medium containing ampicillin plus chloramphenicol, and isopropyl β-thiogalactoside was used to induce transcription of C/EBPβ cDNA. The cells were then lysed and the recombinant C/EBPβ was purified by chromatography on a heparin–Ultrogel column [27]. The purity of the recombinant protein was checked by SDS/PAGE.

DNAase I footprinting experiments

Plasmids pP042 [18] and pP031 [13] carrying the rat AFP promoter and enhancer respectively were labelled with ^{32}P at the *Hin*dIII or *Eco*RI or *Bam*HI site using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase and digested with the appropriate restriction enzyme. The labelled probe was purified by PAGE and electroeluted from the gel, treated with phenol and precipitated with ethanol. DNAase I footprinting experiments were conducted by incubating increasing amounts of partially purified recombinant C/EBPα (a gift from Dr. S. McKnight) or C/EBPβ with 1 ng of the labelled probe for 20 min on ice followed by limited digestion with DNAase I for 45 s at 25 °C [18,28].

Transfection and transient expression experiments

The human hepatoma cells HepG2 were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal calf serum. Chinese hamster ovary (CHO) cells were grown in Ham's F12 medium containing 10% fetal calf serum. Transfection experiments were performed in 6 cm plastic Petri dishes containing about 10^6 exponentially growing cells using the calcium phosphate method. CAT plasmid (2 μg) was routinely used. To prevent artifacts being produced on using single concentrations of the expression vector, co-transfection experiments with a vector allowing expression of a transcription factor were always performed with a wide range of concentrations of each of the expression vectors while keeping the total amount of DNA at 20 μg by adding pUC18 DNA. The precipitate was left in contact with the cells for 4 h. Cells were then subjected to a 20% glycerol shock for 2 min and the medium was changed. After 48 h, cells were lysed by three successive freezing–thawing cycles in 100 μl of 0.25 M Tris/HCl buffer, pH 7.8. The homogenate was centrifuged for 10 min at 11000 ***g*** and the clear supernatant was collected and kept frozen at −20 °C. Protein concentrations were determined by the Bradford method with bovine immunoglobulin (Bio-Rad) as standard.

The CAT reaction was routinely performed on samples containing 25–50 μg of proteins from HepG2 or 100 μg from CHO cells. CAT activity was measured after separation of [^{14}C]chloramphenicol from either the acetyl[^{14}C]chloramphenicol by t.l.c. or the butyryl[^{14}C]chloramphenicol by ex-

traction in tetramethyl-*p*-phenylenediamine/xylene [33]. The two methods gave the same results in our hands. CAT activity of each sample was expressed relative to that of the vector pAFP-CAT or pALB-CAT obtained in the absence of any co-transfected expression vector. Under our conditions, the activity of pAFP-CAT in the HepG2 cells was usually about 8–10% of that of plasmid pSV2-CAT and that of pALB-CAT was 3%. The activity of the promoterless plasmid pBL-UMS-CAT3 was close to the background, less than 0.8% of that of pSV2-CAT, and was not significantly altered by expression of any of the transcription factors used.

Plasmid pCH110 coding for the β-galactosidase (2 μg) was used in some control experiments to monitor the efficiency of transfection. This plasmid was not systematically included in the co-transfection experiments because the activity of pCH110 was greatly affected by the expression of some of the transcription factors. The same was true for plasmid pRSV-βgal.

All the experiments were repeated three to eight times, most often in duplicate, with at least two different preparations of the purified plasmids.

RESULTS

Members of the C/EBP family can bind to the rat AFP promoter and the −2.5 kb enhancer and modulate their activity

C/EBP, now called C/EBPα, was the first member of a new family of transcription factors with a 'leucine zipper' structure to be cloned. A second member was then readily cloned because of its ability to recognize the same site as C/EBPα on the albumin promoter. It was called LAP, now C/EBPβ, and can form heterodimers with C/EBPα. The C/EBP family has now been greatly extended (see ref. [5] for review).

We have used DNAase I footprinting experiments to show that purified recombinant proteins C/EBPα and C/EBPβ both bind to the rat AFP promoter. Two regions of the 200 bp AFP promoter were identified as possible targets for these proteins. The regions protected from digestion by DNAase I were the same for the two proteins, in agreement with the fact that the DNA-binding domains of these proteins are perfectly conserved. Region I (from −120 to −100) is a high-affinity binding site, whereas region II (from −75 to −60) appears to have a lower affinity. These *in vitro* footprinting results are in a good agreement with those obtained with the mouse AFP promoter [7] and with the rat AFP promoter [21].

Our results also indicate that members of the C/EBP family can bind to region 140–173 of the AFP liver-specific enhancer located at −2.5 kb which we previously studied in detail [13]. We had previously shown that this region is protected from DNAase I digestion by proteins from liver nuclear extracts [13].

Figure 1 summarizes the regions of the AFP promoter and enhancer recognized by members of the C/EBP, HNF-1 and NF-1 families.

Because C/EBP dimers can bind to critical regions of the AFP promoter, we evaluated the potential activities of three of the members of the C/EBP family (C/EBPα, C/EBPβ and LIP) on the functioning of the rat AFP promoter. This was achieved by co-transfection experiments in which expression vectors coding for these regulators were co-transfected with constructs bearing the CAT gene under the control of the rat AFP promoter or rat albumin (used as a control). The HepG2 cells used for these experiments are an AFP-producing hepatoma cell line of human origin.

The results indicate that both C/EBPα and C/EBPβ can *trans*-activate the AFP promoter in HepG2 cells (Figure 2). In contrast, LIP, a truncated form of C/EBPβ obtained by an alternative use of a translation initiation site, strongly down-regulated the activity of the AFP promoter (Figure 2).

We extended this study to another transcriptional regulator, DBP, which does not belong to the C/EBP family but is closely related to it. DBP can bind to some of the C/EBP sites and has been cloned using site D of the albumin promoter as a probe to screen a liver cDNA expression library [29]. The expression of

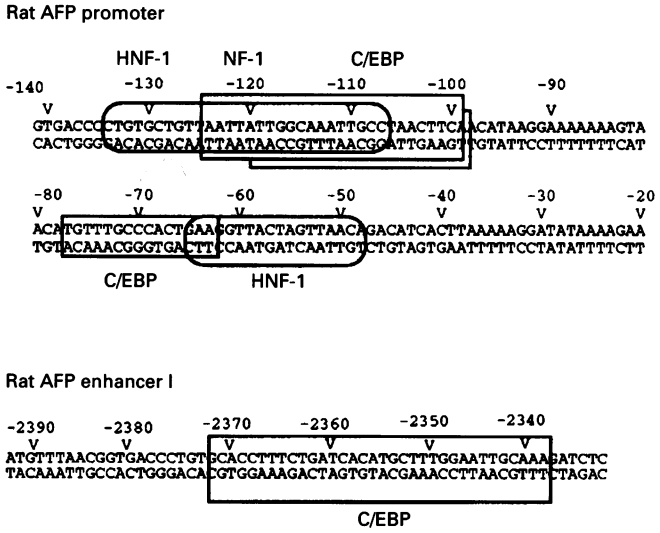

Figure 1 Binding of members of the C/EBP, HNF-1 and NF-1 families to the AFP promoter and enhancer at −2.5 kb

The partial nucleotide sequences of the rat AFP promoter and enhancer at −2.5 kb (ENH1 element) are shown. Numbering of the promoter and enhancer regions is from the transcription start site [14,24]. Boxes C/EBP represent the DNAase I footprints corresponding to binding of purified recombinant C/EBPα and C/EBPβ to the AFP promoter and enhancer. Binding sites previously attributed to NF-1 [18] and HNF-1 [18,40] on the AFP promoter are also surrounded.

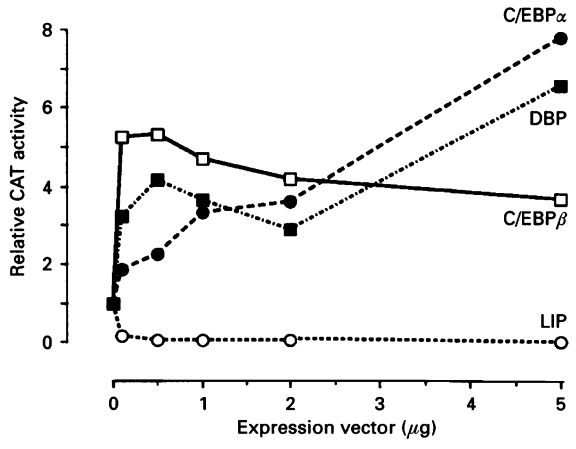

Figure 2 Modulation of rat AFP promoter activity by C/EBPα, C/EBPβ, LIP and DBP in HepG2 hepatoma cells

HepG2 cells were transfected with 2 μg of the plasmid pAFP-CAT and increasing amounts of plasmids pMSV-C/EBP (●), pCMV-LAP (□), pCMV-LIP (○) or pCMV-DBP (■) allowing production of C/EBPα, C/EBPβ, LIP or DBP respectively. The total amount of transfected DNA was kept constant at 20 μg by adding pUC18 plasmid. CAT activities were measured 48 h after transfection. Results are expressed with reference to the activity of the pAFP-CAT plasmid in the absence of any other expression vector.

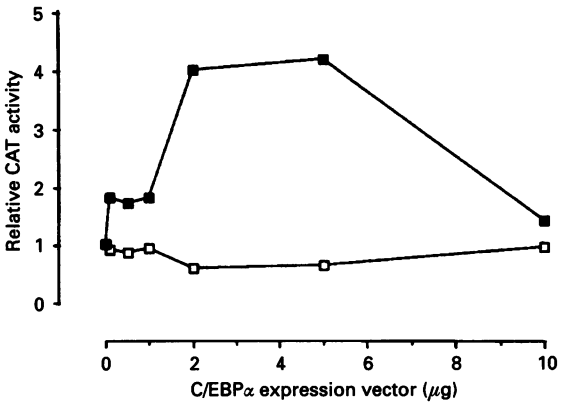

Figure 3 Modulation of the rat AFP enhancer activity by C/EBPα in the HepG2 hepatoma cells

HepG2 cells were transfected with 2 μg of either pBL PO31-tk-CAT (■) or pBL-CAT5 (□) plasmid together with increasing amounts of plasmid pMSV-C/EBP allowing production of C/EBPα. The total amount of transfected DNA was kept constant at 20 μg by adding pUC18 plasmid. CAT activities were measured 48 h after transfection. Results are expressed with reference to the activity of the pBL-PO31-tk-CAT (■) or pBL-CAT5 (□) plasmid in the absence of any other expression vector.

DBP also enhanced the CAT activity driven by the rat AFP promoter in the HepG2 cells (Figure 2).

The controls of all these experiments were parallel experiments with a construct bearing the CAT gene under the control of the albumin promoter (pALB-CAT). Co-transfection of HepG2 cells by the expression vectors and pALB-CAT gave results similar to those obtained with pAFP-CAT: C/EBPα and β, and DBP behaved as activators whereas LIP inhibited the albumin promoter activity (results not shown). The results obtained with pALB-CAT were identical with published data [34] and validated our results with pAFP-CAT.

We also evaluated the functionality of the binding of C/EBP on the AFP enhancer at −2.5 kb. For this, and in order to avoid any participation of the C/EBP-binding sites in the AFP promoter, we constructed and used the plasmid pBL-PO31-tk-CAT5, which contains the 170 bp AFP enhancer at −2.5 kb of the AFP transcription start site in front of the tk promoter to perform co-transfection experiments with the C/EBPα expression vector. C/EBPα stimulated the activity of the AFP enhancer-containing plasmid whereas it had no effect on that of the enhancerless pBL-CAT5 plasmid (Figure 3). This indicated that C/EBPα, on binding to the AFP enhancer, can specifically enhance its activity.

HNF-1α and -1β can act as activators on the AFP promoter in cells of both hepatic and non-hepatic origin

The effect of two members of the HNF-1 family, HNF-1α and HNF-1β (see refs. [1,3,4] for reviews), on the AFP promoter were examined by co-transfecting cells of hepatic (HepG2) and non-hepatic origin (CHO) by vectors allowing their expression together with the pAFP-CAT constructs. These transcription factors, which can bind to and activate several liver-specific promoters, are related to the POU family of transcription factors. They have similar DNA-binding domains and can form heterodimers, but have some quantitative and qualitative differences in their expression in the course of development [3,4,35,36], in their cell specificity and on differentiation of F9 cells [36,37].

Both HNF-1α and HNF-1β activated the rat AFP promoter in HepG2 cells (Figure 4). These results agree with our previous *in vivo* competition experiments indicating that HNF-1 is a key powerful activator of the AFP promoter [19]. They also fit well with the mutation analyses performed by others [12,20,38].

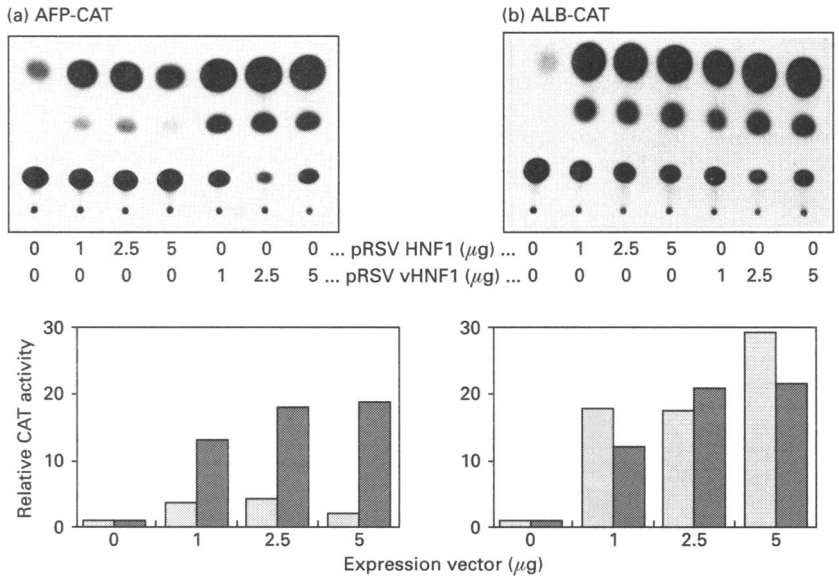

Figure 4 Modulation of the activities of the rat AFP and albumin promoters by HNF-1α and HNF-1β in the HepG2 hepatoma cells

HepG2 cells were transfected with 2 μg of the plasmid pAFP-CAT (**a**) or with 2 μg of the plasmid pALB-CAT (**b**) and increasing amounts of plasmids pRSV-HNF1 or pRSV-vHNF1 allowing production of HNF-1α or HNF-1β respectively. The total amount of transfected DNA was kept constant at 20 μg by adding pUC18 plasmid. CAT activities were measured 48 h after transfection. In the upper part of the figure, they were determined by using t.l.c. In the lower part of the figure they were determined by using extraction with tetramethyl-*p*-phenylenediamine/xylene: ▨, HNF-1; ■, vHNF-1. Results are expressed with reference to the activity of the pAFP-CAT or pALB-CAT plasmid in the absence of any other expression vector.

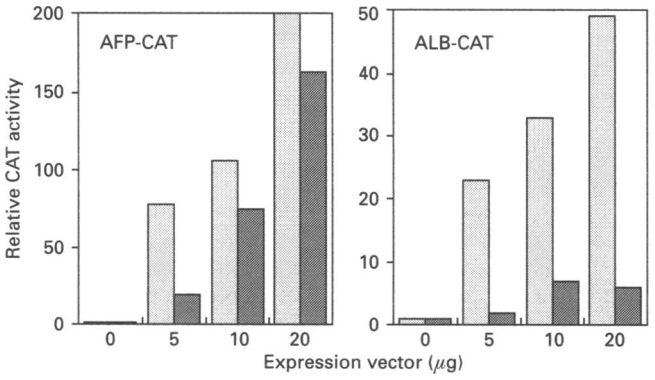

Figure 5 Modulation of the activities of the rat AFP and albumin promoters by HNF-1α and HNF-1β in CHO cells

CHO cells were transfected with 2 μg of either the pAFP-CAT or pALB-CAT together with increasing amounts of plasmids pRSV-HNF1 or pRSV-vHNF1 allowing production of HNF-1α or HNF-1β respectively. The total amount of transfected DNA was kept constant at 20 μg by adding pUC18 plasmid. CAT activities were measured 48 h after transfection. Results are expressed with reference to the activity of the pAFP-CAT or pALB-CAT plasmid in the absence of any other expression vector. ▨, HNF-1; ■, vHNF-1.

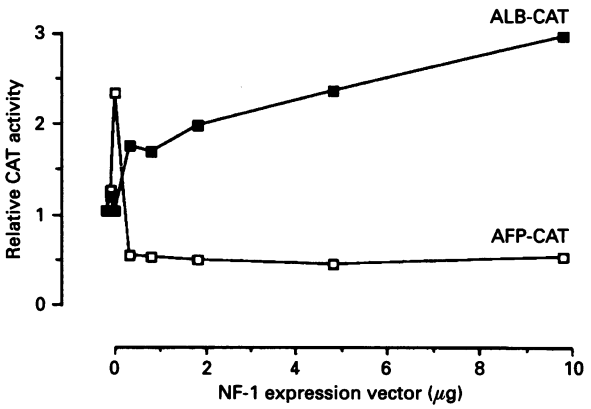

Figure 6 Modulation of the activities of rat AFP and albumin promoters by NF-1 in the HepG2 hepatoma cells

HepG2 cells were transfected with 2 μg of plasmid pAFP-CAT (□) or pALB-CAT (■) with increasing amounts of plasmid pRSV-NF21 which allows production of NF-1. The total amount of transfected DNA was kept constant at 20 μg by adding pUC18 plasmid. CAT activities were measured 48 h after transfection. Results are expressed with reference to the activity of the pAFP-CAT (□) or pALB-CAT (■) plasmid in the absence of any other expression vector.

Perhaps more significantly, our present results show that HNF-1β is more potent than HNF-1α in stimulating the AFP promoter in HepG2 hepatoma cells. This difference between the two members of the HNF-1 family seems to be specific to the AFP promoter, as it was not observed in the parallel experiments on HepG2 cells transfected by the CAT plasmid containing the albumin promoter (pALB-CAT) instead of the AFP promoter (pAFP-CAT) (Figure 4).

The effects of these two factors on the AFP promoter in cells of extrahepatic origin were evaluated in CHO cells. These cells are of ovarian origin and do not produce HNF-1α, HNF-Iβ, AFP or albumin. The construct pAFP-CAT had no significant activity in this cell line (Figure 5), as expected because the AFP promoter is highly liver-specific [11–14]. However, co-transfection of CHO cells with pAFP-CAT plus increasing amounts of the HNF-1α or HNF-1β expression vector resulted in a dramatic increase in the CAT activity, which was proportional to the amount of the HNF-1α or HNF-1β vector over the range used (Figure 5). Thus HNF-1α and HNF-1β can act as powerful activators of the AFP promoter, even in a non-hepatic cell.

NF-1 can play a pivotal role in regulating the AFP promoter activity

The high-affinity site for HNF-1 in the −120 region of the rat AFP promoter, which is crucial for its functioning, partially overlaps that for the ubiquitous factor NF-1. As reported by us and others, there is a very strong footprint in this region of the AFP promoter in DNAase I footprinting experiments with whole nuclear extract from fetal or adult rat liver [18,38,39]. However, it mainly reflects the binding of the very abundant NF-1 protein. This was first revealed by our previous competition experiments using double-stranded DNA fragments carrying NF-1 sites [18]. More recently, we observed that an oligonucleotide covering the high-affinity HNF-1-binding site of the rat albumin promoter (ALB PE) does not alter the footprint obtained with liver nuclear extracts when used as a competitor, even in large excess (F. Richard and J. L. Danan, unpublished work). This indicates that HNF-1 does not significantly contribute to the protection of this region of the AFP promoter under these conditions, in perfect agreement with a very recent report [39]. However, the HNF-1 site at −120 is a much stronger high-affinity binding site than that at −60 [40], and is indispensable for the functioning of the AFP promoter [20]. This suggested to us the possibility of competition between NF-1 and HNF-1 in this functionally important region of the rat AFP promoter.

We therefore examined the role of NF-1 in the functioning of the AFP promoter using an NF-1 expression vector (pRSV-NF21) and the AFP-CAT plasmid to transfect HepG2 cells. NF-1 had a variable effect on the activity of the AFP promoter in HepG2 cells (Figure 6). NF-1 slightly stimulated the AFP promoter activity at low concentrations of the expression vector. At higher concentrations, it strongly reduced the activity of the AFP promoter. This negative effect of NF-1 is specific to the AFP promoter as, in parallel experiments, it slightly stimulated the CAT activity driven by the albumin promoter at all concentrations (Figure 6). Consequently, this negative effect of NF-1 on the AFP promoter activity is not the result of non-specific 'squelching' of the HepG2 cells transcriptional machinery.

DISCUSSION

From this and other studies, it is clear that members of the C/EBP, HNF-1 and NF-1 families can bind *in vitro* to several regions of the rat AFP promoter and enhancer element which governs the expression of the AFP gene and modulate their activity in co-transfection experiments in HepG2 cells.

Members of the C/EBP family are putative regulators with a key role in gene expression in the course of differentiation and development, as is well documented for cells of hepatic and adipogenic origin (see ref. [5] for a review). This is a result of their characteristics. They are expressed in a tightly controlled manner in terms of tissue- and development-specificity; they can use alternative AUG to raise truncated proteins lacking the activating domain and which behave as negative regulators of transcription, as was first shown for LIP [28]. All members can interact between themselves to form homo- and hetero-dimers able to bind DNA because they have a series of leucine residues near a basic region

that is involved in the DNA-binding domain. In addition, they may well interact with other classes of transcription factors and should thus be involved in several cell- or stage-specific regulatory pathways. Hence, this family of transcription regulators is potentially important for modulating gene expression in the liver during development. Indeed, the relative concentrations of these proteins vary greatly during liver development, which could favour the formation of a specific form of homo- or hetero-dimer at a specific stage.

The present study shows that C/EBPα and C/EBPβ are able to activate the AFP promoter in HepG2 cells whereas LIP is a potent negative effector. However, there is no evident correlation between the action of the factors of the C/EBP family we tested on AFP promoter activity in HepG2 cells, their developmental pattern of expression in the liver and transcription rates of the AFP gene in the developing liver. The concentrations of C/EBPα and β proteins increase in liver cells around birth, whereas the LIP/LAP ratio decreases [28,41]. This is the time at which AFP gene transcription falls abruptly [24]. Thus C/EBPα, C/EBPβ and LIP may not directly influence AFP promoter activity in the embryonic liver cell or its down-regulation after birth. However, it is always possible that one of the heterodimers formed by members of the C/EBP family may have a direct or indirect negative effect on the AFP promoter activity in the postnatal period.

By contrast, it is reasonable to think that, *in vivo*, during the late-gestation and postnatal period, C/EBPα directly activates the AFP enhancer at -2.5 kb as it does in the HepG2 cells. This liver-specific element seems to remain functional during adult life [16], and may exert its effect on the albumin promoter located about 27 kb upstream.

The observation that HNF-1β is more potent than HNF-1α in stimulating the AFP promoter in a hepatic cell line may have some physiological relevance to the relative expression of the AFP and albumin genes in the early-embryonic liver. Indeed, HNF-1β is produced before HNF-1α during liver ontogeny [36] and our results show it to be a better activator of the AFP promoter than of the albumin promoter. This correlates well with, and may well account for, the order of appearance and abundance of the AFP and albumin transcripts in the early embryo: AFP gene transcription is earlier and greater than that of the albumin gene at this period of rat development [42,43].

It has also recently been reported that HNF-1β is the only HNF-1-binding activity detected during differentiation of F9 teratocarcinoma cells into endodermal cells, which produce AFP on treatment with retinoic acid [36,37]. This observation is in complete agreement with our findings, and supports our idea that HNF-1β is the major effector of the AFP promoter in early development.

The present study also shows that the relative capacities of HNF-1α and HNF-1β to *trans*-activate may depend on the structure of a promoter and also on the origin of the cell. We indeed observed that HNF-1β is much less active than HNF-1α in CHO (Figure 5) and COS cells (B. Bois-Joyeux and J.-L. Danan, unpublished work) in *trans*-activating the AFP and albumin promoters, in contrast with our results for HepG2 cells. These results correlate well with the recent finding that the action of HNF-1α on the albumin promoter greatly depends on the cell phenotype [44]. Additional or different accessory proteins are probably involved in the *trans*-activation by the HNF-1s in liver cells. Their characterization will represent an important step in understanding how the structure of the promoter and the cellular environment modulate the activity of this class of liver-restricted transcription factors.

Special attention must be paid to the specific negative effect of NF-1 on the activity of the rat AFP promoter. It is probably due to competition between NF-1 and HNF-1 for their overlapping binding sites in the -120 region. Any NF-1 bound to its high-affinity binding site would prevent – or limit – access of the HNF-1 to its binding site in that region, thus reducing the activity of the rat AFP promoter.

Competition between HNF-1 and NF-1 could well be used *in vivo* by the hepatocyte to modulate the activity of the AFP promoter, and hence the transcription rate of the AFP gene during development, carcinogenesis, or in response to hormonal stimuli. The very recent clinical observation that individuals in a family who have hereditary persistence of AFP all have a mutation of a G to an A at -119 in the human AFP promoter [45] supports this hypothesis. The authors also report that the CAT activity of a plasmid containing the mutated AFP promoter is greater than that of a plasmid containing the wild-type promoter in a transient transfection assay with HepG2 cells. They attribute this enhancement of the AFP promoter activity to an increase in the apparent affinity of HNF-1 for the mutated promoter. We believe that the G to A mutation may also lower the affinity for NF-1. The G which is mutated in this region also belongs to the NF-1-binding site in the AFP promoter at a place that plays an important role in the binding of NF-1 to DNA, as indicated by a systematic mutational analysis of NF-1-binding sites [46]. Access of HNF-1s to the mutated AFP promoter would thus be strongly favoured and the activity of the mutant AFP promoter stimulated.

The above data appear to indicate that the modulation of the AFP gene in the course of development which depends, at least in part, on the activity of its promoter may involve some form of competition between HNF-1 and NF-1 for binding to this crucial region. But the modulation is unlikely to depend simply on changes in the HNF-1/NF-1 molar ratio in between the fetal and adult hepatocyte nuclei. Neither gel shift nor footprinting experiments have shown any significant difference in the HNF-1/NF-1 ratio in nuclear extracts from rat livers taken on day 18 of gestation and from adult rats [19]. There may be indirect additional mechanisms that may help – or prevent – one or other of the factors to bind to its target in this region of the AFP promoter. A stage-specific protein might interact directly or indirectly with one of the factors. This protein, different from HNF-1 and NF-1, might recruit one of them to its binding site on DNA via direct protein–protein interaction. Or it might modulate the binding of these factors to the AFP promoter by positive or negative co-operativity. This kind of cross-talk may take advantage of, or induce, the changes in the chromatin structure that occur within the AFP promoter region of liver nuclei between the fetal and adult stage (see ref. [6] for review). Such a change in the chromatin, which favours the binding of NF-1 to DNA, has been well documented in the case of the modulation of the mouse mammary tumour virus promoter by glucocorticoids [47]. Binding of the glucocorticoid receptor induces a change in the position of the nucleosome which allows binding of NF-1, which then stimulates transcription from the promoter. Similarly, NF-1 and changes in the chromatin seem to participate in the mechanism of action of the oestrogen receptor [48].

The hepatocyte can modulate the activity of the promoters and regulatory elements of liver-specific genes in a time-dependent manner in several ways. One is the preferential use of one of the members in a given family of transcription factors. Another may take advantage of the extreme diversity created by possibilities of forming homo- and hetero-dimers with specific functions. A third possibility is synergy or antagonism between factors. Each of these possibilities can themselves be modulated

by the nature and relative concentrations and affinities of each of the different proteins within the families.

Two very recent reports document competition between transcription factors for binding to their overlapping binding sites as a means of modulating the activity of liver-specific *cis*-regulatory elements. One shows that competition between the two liver-specific factors HNF-1 and HNF-3 can be involved in the modulation of the aldolase B promoter activity [49]. The other demonstrates that competition between NF-1 and HNF-3 may participate in the modulation of the albumin far upstream of enhancer activity [50]. Our present data strongly suggest the preferential use of HNF-1β rather than HNF-1α and competition between members of the HNF-1 and NF-1 families to account, at least in part, for the modulation of the AFP promoter activity in liver cells. This provides further information as to how transcription of liver-specific genes can be modulated in response to diverse stimuli in the course of development or carcinogenesis.

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