

Liver enriched transcription factors and differentiation of hepatocellular carcinoma

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Abstract

The development of a complex organism relies on the precise temporal and spacial expression of its genome in many different cell types. The unique phenotype of hepatocytes arises from the expression of genes in a liver specific fashion, which is controlled primarily at the level of mRNA synthesis. By analysing DNA sequences implicated in liver specific transcription, it has been possible to identify members of the nuclear proteins, such as the liver enriched transactivating factors, hepatic nuclear factor 1 (HNF-1), HNF-3, HNF-4, HNF-6, CCAAT/enhancer binding protein (C/EBP), and D binding protein (DBP), which are key elements in the liver specific transcriptional regulation of genes. Each of these factors is characterised by DNA binding domains that bind to unique DNA sequences (cis-acting factors) in the promoter and enhancer regions of genes expressed in terminally differentiated hepatocytes (such as, albumin, α 1-antitrypsin, transthyretin, α -fetoprotein). The determination of the tissue distribution of these factors and analysis of their hierarchical relations has led to the hypothesis that the cooperation of liver enriched transcription factors with the ubiquitous transactivating factors is necessary, and possibly even sufficient, for the maintenance of liver specific gene transcription. With the increase in information about transcriptional regulation, it should be possible to evaluate fully the clinicopathological usefulness of transcription factors in the diagnosis and treatment of hepatocellular carcinoma.

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The tissue specific expression of genes is based on the presence of cis-acting sequences in their promoter and enhancer regions that interact with sequence specific nuclear transcription factors, which potentiate or depress transcriptional initiation.¹ Therefore, the establishment and maintenance of a cell type specific, differentiated phenotype relies on the presence and activity of an array of tissue enriched DNA binding proteins with transactivating activity. In general, the different stages of hepatocyte differentiation have been characterised by the transcripts detected, and the expression of such transcripts is believed to be governed by the sets of transcription factors that are activated.²

Hepatic differentiation is presumed to be the result of the combined action of members of liver enriched transcription factors (LETFs), including CCAAT/enhancer binding protein (C/EBP), D binding protein (DBP), hepatic nuclear factor 3 (HNF-3), HNF-1, HNF-4, and HNF-6. These LETFs regulate each other and form a transcriptional hierarchy that is involved both in the determination and the maintenance of the hepatic phenotype.³ Hepatocarcinogenesis is characterised by the sequential appearance of preneoplastic and neoplastic populations of cells that show, in comparison with their normal counterparts, alterations in their proliferative behaviour and changes in the expression and activity of a large number of liver specific proteins. The phenotypical heterogeneity of hepatocellular carcinoma foci and nodules indicates the existence of distinct subpopulations of lesions.⁵ The promoter regions of several genes, including those encoding albumin, α -fetoprotein (α -FP), α 1-antitrypsin, transthyretin, fibrinogen, and certain members of the cytochrome P450 2C family that are highly expressed in the liver, have been shown to contain consensus sequences that bind different LETFs, and the relative abundance of these factors determines the level of gene expression.^{6–12} Some studies have investigated whether the changes in these LETFs might also occur in hepatocarcinogenesis.

Hepatocyte nuclear factor-1 (HNF-1) family

The HNF-1 family includes HNF-1 α (also called LFB1 or AFP) and HNF-1 β (also called variant HNF-1). These proteins are characterised by a homeobox containing, DNA binding domain that is well conserved throughout evolution and a POU domain that confers sequence specificity. As a result of a similar dimerisation domain in their N-terminal regions, HNF-1 α and HNF-1 β proteins can dimerise on their own to form homodimers or they can form heterodimers with each other. The functions of different transactivation domains localised at their C-terminal regions show that HNF-1 α has a higher potency of transactivation than HNF-1 β (fig 1). HNF-1 α and HNF-1 β genes are located on different chromosomes—chromosomes 12 and 17, respectively.^{13–15} HNF-1 β is expressed early on during embryonic development, in the endoderm of the foregut, whereas HNF-1 α is activated later, upon condensation of the hepatic parenchyma, and its expression decreases in the adult liver (fig 2).^{14–17} Binding sites for HNF-1 have been shown in the promoters or enhancers of genes that are

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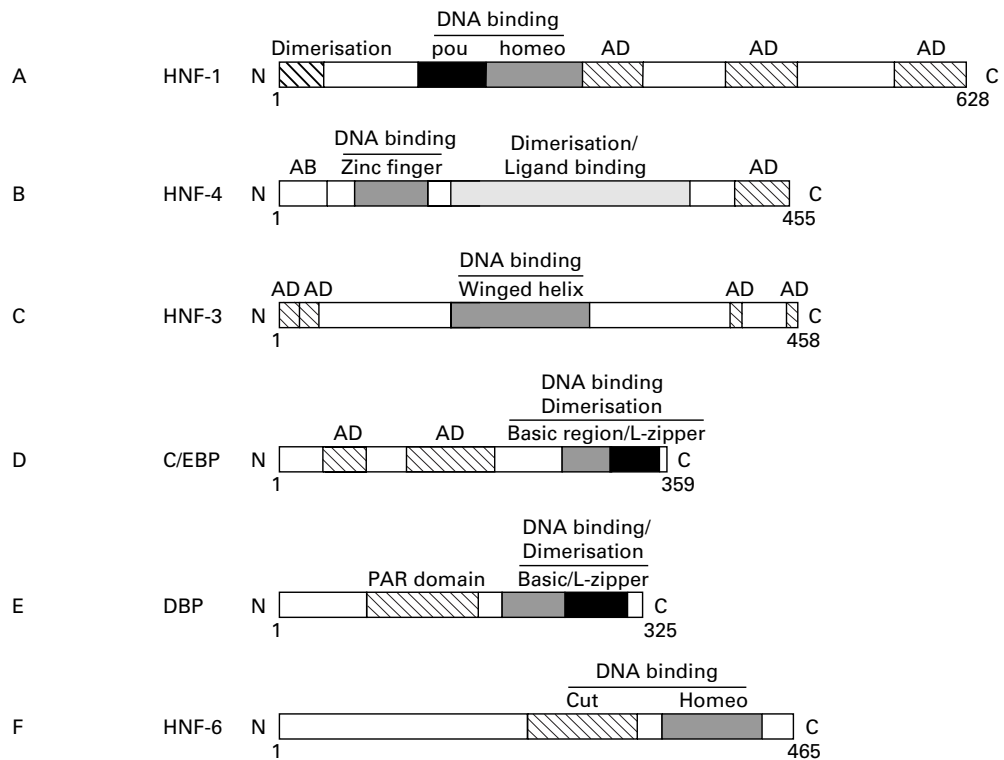


Figure 1 Schematic representation of liver enriched transcription factor proteins. (A) HNF-1 contains highly conserved dimerisation, POU (sequence specific recognition), and homeo domains and non-conserved transcriptional activation domains (AD). (B) HNF4 contains highly conserved DNA binding (zinc finger motif) and dimerisation/ligand binding domains, and non-conserved variant A/B as well as transcriptional activation domains (AD). (C) The HNF-3 family contains a highly conserved DNA binding domain (winged helix motif) and two transcriptional activation domains in the C-terminal and N-terminal regions, respectively. (D) The C/EBP family contains a highly conserved basic region, leucine zipper motif, and two transcriptional activation domains (AD). (E) DBP has a similar structure to C/EBP but it also has a PAR domain. (F) HNF-6 contains two different DNA binding domains: cut and homeo domains.

expressed almost exclusively in the liver, such as albumin,⁶ α -fetoprotein,⁷ α -fibrinogen, β -fibrinogen, α 1-antitrypsin,⁹ transthyretin,¹⁰ aldolase B,¹⁸ and the hepatitis B virus large surface protein.¹⁹ The study of the rat hepatoma cell, H4II, and its derivatives show that HNF-1 α expression parallels the hepatic phenotype of the cells. HNF-1 α is expressed only in fully differentiated cells and is absent in dedifferentiated variants or extinguished somatic hybrids. On the other hand, HNF-1 β retains its expression in dedifferentiated variants and somatic cell hybrids.^{20–21} In hepatocarcinogenesis, the transcriptional alterations of HNF-1 can be demonstrated by the reverse transcription–polymerase chain reaction (RT–PCR)²² and the RNA protection assay,²³ revealing that the HNF-1 α :HNF-1 β ratio is higher in well differentiated human hepatocellular carcinoma and lower in poorly differentiated human hepatocellular carcinoma, the latter being lower by 20–30%; in addition, HNF-1 β mRNA remains unchanged in mouse liver tumours. The concentration of HNF-1 α protein is also reduced in rat liver tumours and HNF-1 binding activity is reduced by lowered concentrations of HNF-1 α protein in the transition from well differentiated to poorly differentiated human hepatocellular carcinoma.^{24–25} Both HNF-1 α and HNF-1 β are also found in other tissues including kidney, stomach, and intestine. The loss of HNF-1 α has been shown during renal carcinogenesis, which is usually

accompanied by dedifferentiation processes, including the loss of tissue specific gene expression.²⁶ The promoter analysis of HNF-1 α shows that in addition to HNF-1 α autoregulation, HNF-4 is an essential activator of HNF-1 α gene expression (fig 3).

Hepatocyte nuclear factor 4 (HNF-4)

HNF-4 is expressed in liver, kidney, and intestine in the adult,²⁷ and activates a diverse set of liver genes such as transthyretin and α 1-antitrypsin²⁸ in early liver development, often interacting in synergy with adjacent binding factors.²⁹ It belongs to the orphan steroid hormone nuclear receptor superfamily and is characterised by two highly conserved domains (fig 1). The DNA binding domain in the N-terminal half consists of two “zinc finger” motifs followed by an extensive C-terminal ligand binding domain, which performs a variety of functions, including transactivation, ligand binding, and protein dimerisation.²⁷ During mouse development, HNF-4 is expressed in the primary endoderm at 4.5 days and then restricted to the visceral endoderm from 5.5 days (fig 2). In cell genetic analysis of hepatocyte differentiation, HNF-4 shows a capability to transactivate endogenous HNF-1 α and liver genes such as α 1-antitrypsin and to induce redifferentiation of a dedifferentiated hepatoma cell line, H5, by stable transfection of exogenous HNF-4.³⁰ H5 cells express neither HNF-1 α nor HNF-4; however,

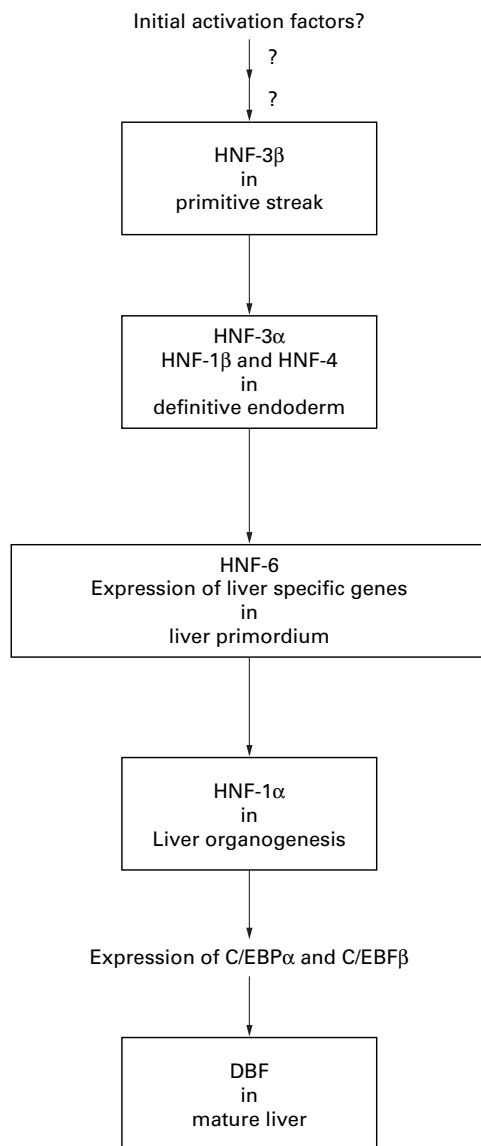


Figure 2 Hierarchy of expression of liver enriched transcription factors (LETFs) in liver development. Boxes indicate the sequential developmental stages.

the presence of C/EBP α and HNF-3 has been revealed, implying that these factors alone are not sufficient to maintain expression of marker genes of hepatic differentiation. After HNF-4 transduction, cells express the previously silent HNF-1 α and show activation of some hepatic proteins, but not of the endogenous HNF-4. This study implies a transcriptional hierarchy from HNF-4, to HNF-1 α , to target genes (fig 3). Furthermore, the effects of HNF-4 expression extend to the re-establishment of differentiated hepatic epithelial cell morphology,³¹ such as inducing: (1) re-expression of E-cadherin, which is a prerequisite for the formation of the junctional complex, including desmosomes and tight junctions; (2) simple epithelial polarity; (3) slow, "pile up" growth habit. It is reported that a factor, "seven up", which is the drosophila homologue of HNF-4, is involved in terminal fat cell differentiation in drosophila.³² These data indicate that the HNF-4 gene might be a tumour suppressor gene that plays important roles in differentiation and antipro-

liferation. The expression of HNF-4 decreases during hepatocarcinogenesis and a coordinate variation of HNF-4 and HNF-1 α binding activity have been demonstrated.²⁴⁻³³ The upstream regulation of HNF-4 is still unclear, although HNF-4 transactivation is blocked by the intracellular accumulation of the HNF-1 α protein (fig 3).³⁴ Experiments have shown that the tyrosine or serine/threonine phosphorylations of HNF-4 negatively modulate its DNA binding and transactivation,³⁵⁻³⁶ and results suggest that HNF-4 might be regulated during cellular growth. The action of HNF-4, an orphan receptor, might be influenced by a potential ligand (fig 3).

C/EBP and DBP

C/EBP family members have a basic region and an adjacent leucine zipper (b/ZIP) structure, and they all bind to similar DNA sequences (fig 1).³⁷⁻³⁸ In this family, C/EBP α , C/EBP β and C/EBP δ homodimerise or heterodimerise, and have different activation effects. They show strong similarity in their C-terminal sequences and divergence in their N-terminal transactivation domains.³⁸⁻³⁹ C/EBP proteins are expressed at a later stage in development (fig 2), and are found abundantly in liver and fat tissues, particularly in fully differentiated cells.⁴⁰⁻⁴³ Transduction of C/EBP α cDNA into pre-adipocytes results in the suppression of clonal cell growth and the promotion of adipogenic differentiation by inducing the expression of adipocyte specific genes such as 422 adipose P2 protein 422.⁴⁴⁻⁴⁶ Proof that the expression of C/EBP α proteins is required for pre-adipocyte differentiation is also provided by using the antisense RNA approach.⁴⁷⁻⁴⁸ Constitutive antisense C/EBP α RNA impairs the expression of C/EBP α itself and of adipocyte specific genes. C/EBP α is also expressed strongly in the mature hepatocyte and stimulates the transcription of liver specific genes such as albumin,⁴⁹⁻⁵⁰ transthyretin, and α 1-antitrypsin.⁵¹ C/EBP α transcription correlates closely with the passage of hepatocytes through the cell cycle of the regenerating liver.⁵² Proliferating hepatocytes in the partially hepatectomised liver demonstrate an abrupt reduction of C/EBP α mRNA within the first three hours of surgery, corresponding to the transition from the G0 to the G1 phase of the cell cycle. C/EBP α returns to a normal level by 72 hours, through a gradual increase after the S phase. All evidence indicates that C/EBP α regulates two aspects of terminal differentiation: induction of differentiation specific genes and cessation of mitotic growth. One mechanism of C/EBP α -induced growth arrest is that C/EBP α upregulates p21/SDI-1 (senescent cell derived inhibitor 1) gene expression and stabilises the post-translational p21/SDI-1 protein.⁵³ p21/SDI-1 interacts with cyclin dependent kinases (CDKs), cyclins, and proliferating cell nuclear antigen (PCNA) to inhibit the activity of CDKs, and prevents DNA synthesis by inhibiting DNA polymerase. The C/EBP family plays an important role in both the inhibition of proliferative and its establishment, as well as in maintaining the differentiation of

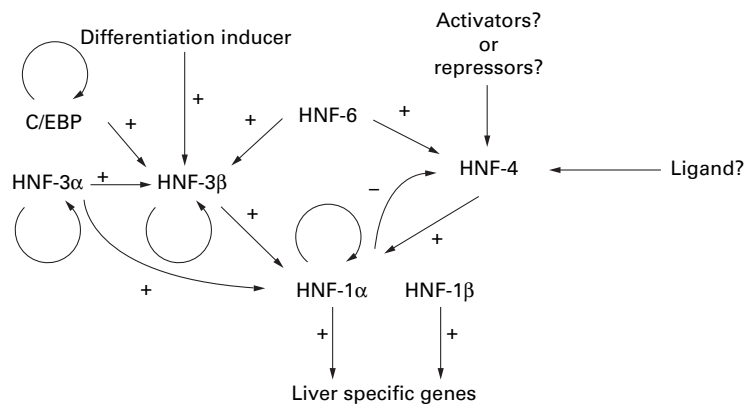


Figure 3 Regulatory network of liver enriched transcription factors. The circular arrows represent autoregulation.

tissues in which C/EBPs are found. In human hepatocellular carcinoma tissues, the level of C/EBP α expression is very low or undetectable.^{24 54} Induction of C/EBP α expression in human hepatoma cells, Hep3B and HepG2, results in reversible arrest of proliferation, and delayed tumorigenesis is seen in immunodeficient mice implanted with two transfected cells.⁵⁵ C/EBP β (also called NF-IL6) and C/EBP δ are also mediators that regulate the acute phase response and they have low activity until activated by inflammatory stimuli. Activated C/EBP β can transactivate multiple cytokine genes⁵⁷⁻⁵⁹ and promote the differentiation of macrophages and granulocytes.^{60 61} Another member of the b/ZIP family, DBP, binds to the D element of the albumin promoter that is recognised by C/EBP β .⁶² Both DBP and C/EBP β , as liver enriched transcription factors, appear to be involved in the differentiation and regulation of acute phase and immune responses; however, DBP belongs to a distinct b/ZIP family, the PAR protein family, and is detected only after birth (fig 2).⁶³ DBP and C/EBP β do not heterodimerise, and C/EBP β has a more relaxed binding specificity.^{64 65}

Hepatocyte nuclear factor 3 (HNF-3)

The HNF-3 family comprises three proteins, HNF-3 α , HNF-3 β , and HNF-3 γ . These proteins share high homology in the winged helix/fork head DNA binding domain and in two short similar regions in their C-terminal and N-terminal regions, which exhibit transactivity (fig 1).⁶⁶⁻⁶⁸ They have been shown to be required for node and notochord axis formation and endodermal differentiation in the mouse embryo (fig 2).^{69 70} HNF-3 β , HNF-3 α , and HNF-3 γ are activated sequentially during development. HNF-3 β mRNA is expressed first in the primitive streak and the node before the expression of any liver genes. Slightly later than HNF-3 β , HNF-3 α (along with HNF-3 β) is expressed in the definitive endoderm that lines the developing gut and subsequently forms components of the liver, lung, pancreas, and alimentary canal. HNF-3 γ is expressed in the early liver and more posterior endoderm after the gut has formed.⁷¹ In combination with other liver enriched transcription factors that are expressed later, HNF-3 binds and transactivates numerous liver specific genes such as

albumin, transthyretin, α 1-antitrypsin,^{67 72} and transcription factor HNF-1 α (fig 3).⁷³ The concentration of HNF-3 α increases when hepatocyte derived cell lines are cultured on an extracellular matrix gel substratum. Extracellular matrix gel substrata coordinately induce differentiated cell morphology and liver gene transcription in primary hepatocyte cultures.^{74 75} Thus, HNF-3 α is important in transducing extracellular signals in the maintenance of hepatocyte differentiation. A winged helix gene in *Caenorhabditis elegans*, lin-31, appears to act via a receptor tyrosine kinase signal transduction cascade.⁷⁶ Once HNF-3 α and HNF-3 β genes are activated, the gene products help to maintain their own synthesis by autoactivation (fig 3).^{77 78} In primary cultured rat hepatocytes that exhibit the transition between growth and differentiation, the constant expression of HNF-3 α and HNF-3 β is seen; nevertheless, HNF-1 α , HNF-4, C/EBP α , and C/EBP β show a decrease during proliferation and an increase after the induction of differentiation.⁷⁹ In chemically induced mouse liver tumours, the expression of HNF-3 α and HNF-3 β remains unchanged, although that of HNF-3 γ increases.²³ It is evident that HNF-3 α and HNF-3 β expression is necessary to maintain basic hepatocyte function. In addition, HNF-3 α and HNF-3 β also mediate the cell specific transcription of genes that are important for the function of intestine, stomach, and pancreatic acinar cells from the foregut. The HNF-3 family is expressed at earlier stages of embryonic development and is competent for transactivation even in dedifferentiated hepatocyte derived cells, when other liver transcription factors are inactive or absent. Thus, members of the HNF-3 gene family might be the primary factors in the hierarchy of the expression of liver enriched transcription factors in hepatogenesis (fig 2).

Hepatocyte nuclear factor 6 (HNF-6)

HNF-6 is a recently identified member of the family of liver enriched transcription factors.^{80 81} It contains two different DNA binding domains: the novel homeodomain and a domain homologous to the drosophila cut domain (fig 1). HNF-6 is expressed in tissues that originate from the endoderm cells lining foregut, liver, pancreas, nervous system, brain, and spinal cord.^{82 83} In the study of HNF-6 potential target genes during development, it has been found that there are HNF-6 binding sites in the promoter regions of HNF-3 β and HNF-4, as well as in the liver specific genes transthyretin and α -FP.⁸¹ An overexpression of HNF-6 can stimulate the expression of HNF-3 β and HNF-4 (fig 3) in cotransfection experiments. The onset of HNF-6 gene transcription is detected in the liver at embryonic day 9, correlating with the onset of liver differentiation (fig 2). In situ hybridisation studies of staged specific embryos demonstrate that the HNF-6 expression pattern and level are consistent with those of its target gene HNF-3 β in liver and pancreas. HNF-6 expression disappears transiently from the liver between embryonic days 12.5 and 15, but is

present again in the liver after embryonic day 15.⁸² This pattern is paralleled by HNF-3 β . In addition, HNF-6 and HNF-3 β transcripts are expressed abundantly and colocalise in the exocrine acinar cells of the pancreas on day 18 of gestation and in the adult liver, although their expression patterns diverge in pancreatic epithelium.⁸³ These findings suggest that HNF-6 might regulate hepatocyte specific genes and play a role in epithelial differentiation of the the gut endoderm by acting on HNF-3 β .

Conclusion and perspectives

The progress made to date suggests strongly that the combined control of liver specific gene transcription and a regulatory network among liver enriched transcription factors are involved in the fine tuning of hepatocyte differentiation. A variety of pathophysiological states in the liver are represented by abnormalities in hepatic protein synthesis. Many human hepatocellular carcinomas develop from precancerous states to carcinoma by multistep carcinogenesis, which is considered to be involved in the complex alteration of proteins.⁸⁴ The molecular mechanism involved in the development of hepatocellular carcinoma remains almost unknown. The histopathological features of hepatocellular carcinoma tissues are useful in evaluating their clinical progress and predicting patients' prognosis: well differentiated hepatocellular carcinoma is usually less than 2 cm in diameter, and rarely metastasises; some of the less well differentiated hepatocellular carcinomas are believed to develop from a clone within a well differentiated hepatocellular carcinoma nodule, morphologically referred to as a "nodule in nodule", and have a strong metastatic potential.⁸⁴⁻⁸⁶ When some of the regulatory molecules are not present in sufficient amounts, the gene that requires that set of transcription factors is either not activated or maintains low rates of expression. We have shown that the C/EBP α gene in hepatocellular carcinoma cells can induce a cascade of transcriptional events to alter the phenotype and redifferentiate cancer cells into a more normal phenotype, or to induce cytostatic growth arrest. Advances in molecular and genetic biology have made it possible to introduce exogenous nucleic acids into target cells and to alter their genetic make up. An obvious area of potential clinical application of these new technologies is the treatment of genetic and acquired disorders. We believe that inducing the expression of HNF-4 and HNF-1 α should be an important strategy for inducing redifferentiation of less well differentiated hepatocellular carcinoma into a well differentiated one. Further efforts should be directed to elucidate how the liver enriched transcription factors work together. Amelioration of liver dysfunction can be achieved by inducing or intervening in genetic regulatory pathways that control hepatocyte differentiation.

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