

Expression of Hepatic Transcription Factors during Liver Development and Oval Cell Differentiation

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Abstract. The oval cells are thought to be the progeny of a liver stem cell compartment and strong evidence now exists indicating that these cells can participate in liver regeneration by differentiating into different hepatic lineages. To better understand the regulation of this process we have studied the expression of liver-enriched transcriptional factors (HNF1 α and HNF1 β , HNF3 α , HNF3 β , and HNF3 γ , HNF4, C/EBP, C/EBP β , and DBP) in an experimental model of oval cell proliferation and differentiation and compared the expression of these factors to that observed during late stages of hepatic ontogenesis.

The steady-state mRNA levels of four (HNF1 α , HNF3 α , HNF4, and C/EBP β) "liver-enriched" transcriptional factors gradually decrease during the late period of embryonic liver development while three factors (HNF1 β , HNF3 β , and DBP) increase. In the normal adult rat liver the expression of all the transcription factors are restricted to the hepatocytes. However, during early stages of oval cell proliferation

both small and large bile ducts start to express HNF1 α and HNF1 β , HNF3 γ , C/EBP, and DBP but not HNF4. At the later stages all of these factors are also highly expressed in the proliferating oval cells. Expression of HNF4 is first observed when the oval cells differentiate morphologically and functionally into hepatocytes and form basophilic foci. At that time the expression of some of the other factors is also further increased.

Based on these data we suggest that the upregulation of the "establishment" factors (HNF1 and -3) may be an important step in oval cell activation. The high levels of these factors in the oval cells and embryonic hepatoblasts further substantiates the similarity between the two cell compartments. Furthermore, the data suggest that HNF4 may be responsible for the final commitment of a small portion of the oval cells to differentiate into hepatocytes which form the basophilic foci and eventually regenerate the liver parenchyma.

DIFFERENTIATION as well as other basic cellular processes are influenced by humoral (Houart et al., 1990; Nakabayashi et al., 1989), intercellular (Fraslin et al., 1985), and matrix-cellular (DiPersio et al., 1991; Liu et al., 1991) interactions. However, the fundamental mechanism responsible for phenotypic diversity is the expression of a set of genes specific for a particular cell type. The differences in gene expression between cell types are mostly determined by transcriptional regulation (Alberts et al., 1989) using a specific combination of transcriptional or *trans*-acting factors.

A number of transcription factors that are enriched in but not exclusive to the mature hepatocyte have been identified and cloned (for review see Johnson, 1990; Lai and Darnell, 1991; DeSimone and Cortese, 1992; Crabtree et al., 1992). These factors as well as ubiquitously expressed ones bind to multiple promoters and enhancers of target genes to regulate

liver-specific gene transcription. Presently, four major families of "liver-specific" transcription factor families have been described. HNF1 α and HNF1 β , members of the HNF1 family are distantly related to the homeobox proteins (Lichsteiner and Schibler, 1989; Baumhueter et al., 1990; Mendel et al., 1991a; Courtois et al., 1988; Frain et al., 1989; Kuo et al., 1990a). HNF3 proteins (α , β , and γ) belong to the "forkhead" protein gene family (Lai et al., 1990, 1991). Recently several new members of this family have been described based on sequence homology (Clevidence et al., 1993). HNF4 is a member of the nuclear steroid-thyroid receptor superfamily and contains a zinc finger DNA binding domain (Sladek et al., 1990). C/EBP is the original leucine zipper protein (Landschulz et al., 1988, 1989). Other members (Williams et al., 1991) of this gene family such as C/EBP β (Descombes et al., 1990; Poli et al., 1990) and DBP (Mueller et al., 1990) are also important in hepatocyte gene regulation.

In recent years there has been a tremendous development in the understanding of transcriptional regulation (McKnight and Yamamoto, 1992). However, most of these studies have

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examined the regulation of single genes at the cellular or molecular level while only a few attempts to find a connection between transcriptional factors and cellular reactions *in vivo* have been reported (Kuo et al., 1992; Griffo et al., 1993). Since practically every gene is regulated by several *trans*-acting factors, and each transcriptional factor drives several genes, there is very little chance that one factor would direct the complex process of cell differentiation. However it is likely that a coordinated action of a battery of transcriptional factors is responsible for the complex changes in differential gene expression.

During embryonic development in the rat, the liver consisting of cords of primitive hepatocytes, the hepatoblasts, emerges from the primitive foregut as a small bud at day 9 after conception. As development progresses the hepatoblasts differentiate into fully mature hepatocytes and also give rise to the epithelium comprising the bile ducts of the liver. In the adult rat certain experimental conditions can be used to induce the proliferation of a distinct population of small epithelial cells in the liver (Farber, 1956; Lemire et al., 1991). These cells, conventionally known as oval cells, are characterized by oval nuclei and basophilic cytoplasm (Farber, 1956), and show features of both bile duct cells and fetal hepatocytes (Lemire et al., 1991; Evarts et al., 1987*b*, 1990). In two of the experimental models it has been conclusively demonstrated that oval cells are capable of differentiation into hepatocytes (Lemire et al., 1991; Evarts et al., 1987*a*). Although oval cells are morphologically very similar, they exhibit heterogeneous functions (Fausto et al., 1987). Most of them express albumin, but they also produce α -fetoprotein (AFP) (Evarts et al., 1987*a,b*), an oncofetal protein expressed in the hepatoblasts in the embryonic liver. However, the developmental potential of oval cells is not confined to hepatic lineages, as they have been shown to differentiate into glandular intestinal-type epithelium, and have been implicated in the development of pancreatic-type tissue (Lemire et al., 1991; Evarts et al., 1987*a*; Tatematsu et al., 1985; Kimbrough et al., 1972; Rao et al., 1986). The observations that subpopulations of proliferating oval cells phenotypically resemble hepatoblasts, and that the oval cells originate in or around the ductal structures in the portal areas, have lent support to the hypothesis that these structures harbor a cell compartment capable of functioning as facultative stem cells whenever parenchymal cells are unable to proliferate in response to growth stimuli (Thorgeirsson and Evarts, 1990; Sigal et al., 1992; Shiojiri et al., 1991; Sell, 1993).

In an attempt to better understand the role of the different transcription factors in the regulation of hepatocyte differentiation we have studied the cellular expression of liver-specific transcription factors during late stages of liver ontogeny and during oval cell proliferation and differentiation in the adult liver of the rat. First, we examined the steady state level of mRNA for the HNF1, HNF3, HNF4, C/EBP, and DBP transcriptional factors by northern blot analysis from the 16th day of embryonic development. Second, we used the combination of partial hepatectomy and feeding of 2-acetylaminofluorene (AAF) to initiate proliferation and differentiation of oval cells in the adult rat liver (Evarts et al., 1987*b*). In this experimental model the first oval cells appear

1. *Abbreviations used in this paper:* AAF, 2-acetylaminofluorene; AFP, α -fetoprotein.

in the periportal region \sim 2 d after partial hepatectomy and gradually invade the whole liver lobulus. The peak of oval cell proliferation occurs \sim 7–10 d after the operation. During the period of oval cell proliferation, basophilic foci consisting of small hepatocytes are formed by differentiation of oval cells and the liver parenchyme is gradually reconstructed. This reconstruction is usually complete at day 16 after partial hepatectomy. We have hypothesized that the differentiation program of oval cells in the adult liver might mimic that of early embryonic hepatoblasts. In the present study the changes in the expression of the steady state mRNA levels of the different nuclear factors were studied by Northern blot analysis throughout the period of oval cell proliferation and differentiation. To determine the cellular localization of mRNA transcripts for the different nuclear factors during the complex process of oval cell differentiation we used *in situ* hybridization analysis.

Although we were unable to demonstrate clear sequential changes in the mRNA expression level of the different factors in either of the models by Northern blot analysis, the temporal and spatial distribution of the transcripts as assessed by *in situ* hybridization indicate that the factors are regulated individually and may play crucial roles at different stages of hepatocyte development.

Materials and Methods

Animal Models

Proliferation and differentiation of oval cells in the adult rat liver was achieved as previously described (Evarts et al., 1987*b*) by administration of AAF to male Fischer 344 rats (150 g) by gavage five times over a week, at the end of which a two-third partial hepatectomy was performed. After 1-d recovery, AAF administration was continued for 4 d. The total dose of AAF was 9 mg/rat. Animals used in the AAF experiments and the pregnant or newborn rats were sacrificed at the times indicated. All animals were sacrificed in the late afternoon because of the known diurnal variation in the expression of at least one of the studied transcriptional factors, DBP (Wuarin et al., 1992).

Sections of the livers were fixed for morphological examination and *in situ* hybridization as described below. The remaining liver tissue was quickly frozen in liquid nitrogen for later extraction of RNA.

RNA Preparation and Northern Analysis

Poly(A)⁺RNA from liver tissue was obtained by oligo(dT)-cellulose chromatography of total RNA by extraction of quick-frozen tissue in guanidine isothiocyanate, sedimentation of total RNA through a cesium chloride cushion (Schweizer and Goertler, 1980). Poly(A)⁺RNA was separated by electrophoresis in 1% agarose/2.2 M formaldehyde gels and transferred onto a nylon membrane (MSI, Westboro, MA). Blots were hybridized overnight at 42°C with ³²P-labeled probes. An adequate control probe in these experiments could not be found since the expression of standard control genes such as GAPDH and actin were affected by the protocol (Bisgaard et al., 1994). Equal loading was assessed by staining of agarose gels with ethidium bromide. The following probes were used: rat HNF1 α (Su yan Liu, National Cancer Institute, Bethesda, MD); mouse HNF1 β (Gerald R. Crabtree, Stanford University, Stanford, CA); rat HNF3 α,β,γ and 4 (William S. Chen, The Rockefeller University, New York); rat C/EBP β and DBP (Ueli Schibler, University of Geneva, Geneva, Switzerland); C/EBP (Steven L. McKnight, Howard Hughes Research Laboratories, Baltimore, MD).

In Situ Hybridization Analysis

The *in situ* hybridizations were performed on formalin fixed tissue sections as described by Wright et al. (1991). In brief, liver tissue was fixed in 10% neutral formaldehyde, paraffin embedded, and tissue sections cut 5- μ m thick. After deparaffinization, sections were permeabilized for 10 min in PBS with 5 mM MgCl₂ followed by treatment for 10 min with 0.2 M Tris

(pH 7.6) containing 0.1 M glycine. The sections were then fixed for 20 min in 4% paraformaldehyde followed by treatment with 1 mg/ml proteinase K (ICN Radiochemicals, Irvine, CA) in PBS for 8 min and refixed for 5 min in 4% paraformaldehyde. The sections were then prehybridized for 4 h at 45°C followed by hybridization with ³²P-labeled (Amersham Corp., Arlington Heights, IL) riboprobes for 16 h. The composition of the hybridization mixture was as described by Wright et al. (1991). The sections were washed at increasing stringency, processed for autoradiography and exposed for 3–4 wk. Serial sections were hybridized with the sense strand of the applied RNA probe as negative control (Figs. 4 B, 6 C, and 9 D).

Results

Expression of Transcriptional Factors during Embryonic Development

We analyzed the expression of liver enriched transcriptional factors by northern analysis from day 16 of embryonic age at which time the liver is easily dissected out of the embryos (Fig. 1). The levels of albumin expression were very similar at the time points studied showing that it had already reached a maximal level by day 16 of embryonic age. The transcriptional factors could be divided into three groups based on the pattern of expression: (a) HNF3 γ and C/EBP expression appeared to be relatively constant throughout the late gestational period. C/EBP expression increased slightly around birth, consistent with previous reports (Birkenmeier et al., 1989), while that of HNF3 γ remained constant. (b) HNF1 α ,

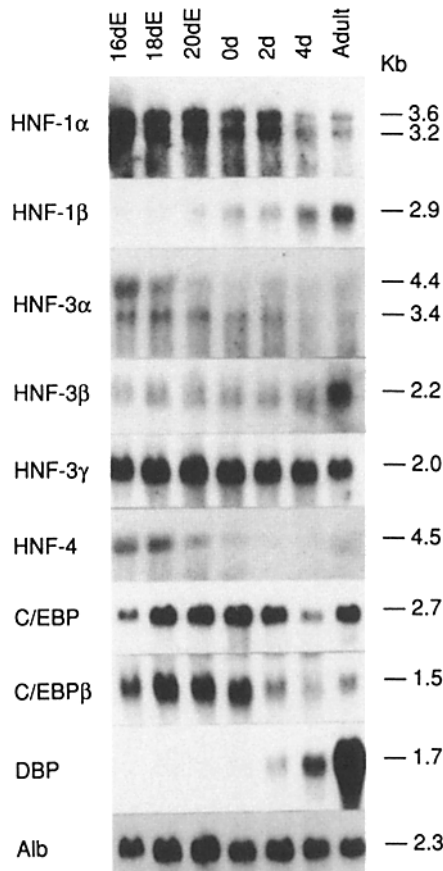


Figure 1. Northern blot analysis of the liver-enriched transcriptional factors in the liver from 16th gestational day (16dE).

HNF3 α , HNF4, and C/EBP β showed a gradual decrease in expression during the late gestational period. While the expression of C/EBP β similar to C/EBP was highest around birth, the other factors reached maximal level of expression at day 16 to 18 of embryonic age. For HNF1 α , the steady-state levels of the two transcripts of 3.6 and 3.2 kb behaved similarly. For HNF3 α the steady-state levels of the 4.4-kb transcript decreased earlier than the 3.4-kb form. The HNF4 transcript was detected at very low levels after birth but a longer exposition time clearly revealed the presence of the transcripts. (c) The steady-state levels of HNF1 β , HNF3 β , and DBP mRNA showed a gradual increase, with the highest levels detected in the adult liver. Transcripts for DBP were only detected after birth. It is noteworthy that in two gene families (HNF3 and C/EBP) all three expression patterns were present. Furthermore, the two members of the HNF1 family also showed different expression patterns. This suggests that during liver development members of the gene families are individually regulated and different members of the same family may be important in specific steps of the maturation process (Tripodi et al., 1991).

Expression of Transcriptional Factors during Oval Cell Proliferation and Differentiation

The overall expression pattern of the different hepatocyte nuclear factors during the proliferation and differentiation of oval cells in the adult rat liver as assessed by northern analysis was distinctly different from the pattern observed during embryonic development (Figs. 1 and 2). The steady-state levels of HNF1 α and HNF1 β increased gradually, with the highest levels detected between days 7 and 13 after partial hepatectomy (Fig. 2). This is the time period where the oval cell proliferation reaches a maximum and foci of basophilic hepatocytes are formed by differentiation of oval cells.

The expression pattern of the three members of the HNF3 family was somewhat different from those of the other families. While the HNF3 α expression was highest at the later time points (day 13 to 16), HNF3 β and HNF3 γ steady-state levels reached their maximum around day 9, and thereafter gradually decreased to near control levels at day 16. For both C/EBP β and DBP the Northern blot analysis revealed increased steady-state levels of their transcripts throughout the experimental period, while the steady-state levels of HNF4 and C/EBP appeared to be unchanged when compared to the time of partial hepatectomy (day 0) (Fig. 2).

To better understand the function of the different nuclear factors in the complex sequence of events involved in the proliferation of oval cells and their role in reconstitution of the liver parenchyma, we used in situ hybridization with radiolabeled riboprobes to examine the cellular distribution of the RNA transcripts for the transcription factors. To simplify the presentation of our results we have chosen to summarize the relative distribution of all the factors examined for the individual epithelial cell types thought to be derived from differentiation of oval cells as well as in the "old" hepatocytes. These expression patterns are summarized in Table I.

In the normal adult rat liver all the transcription factors studied were expressed in the hepatocytes but not in the bile epithelium (Table I). The spatial distribution of the transcripts from different factors was not always equal throughout the liver acinus. For example, HNF1 α and DBP tran-

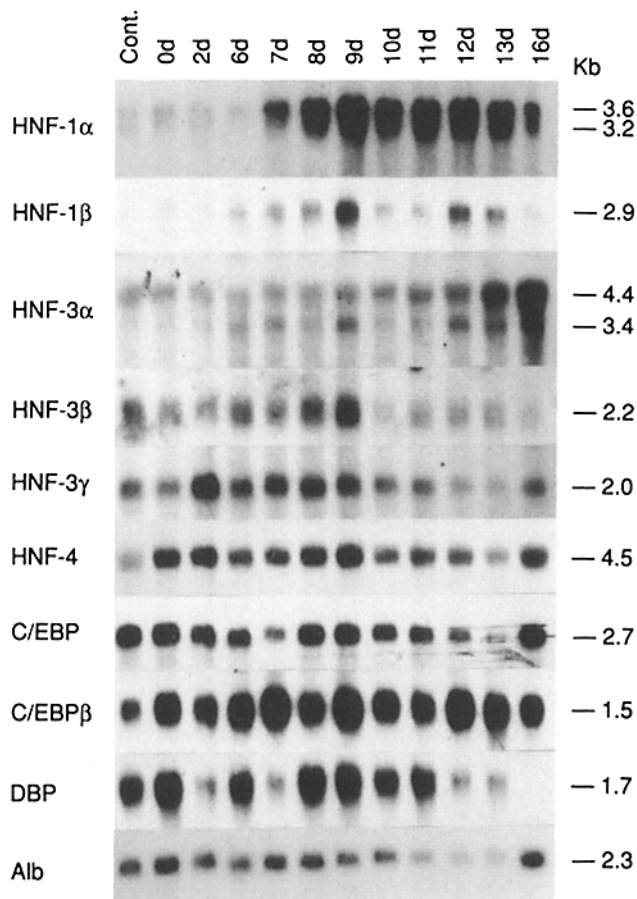


Figure 2. Northern blot analysis of the liver-enriched transcriptional factors during oval cell proliferation and differentiation in the AAF-partial hepatectomy model (see Materials and Methods). 0 d is the day of partial hepatectomy.

scripts were distributed evenly throughout the liver acinus while HNF1 β , HNF3 γ , and C/EBP β transcripts accumulated at slightly higher levels in the hepatocytes closest to the portal veins, and HNF4 and C/EBP transcripts showed a mild preference of the hepatocytes located in the areas closest to the central veins (data not shown). Such a zonal distribution has previously been reported for C/EBP (Kuo et al., 1990b; Moorman et al., 1991).

Table 1. Distribution of In Situ Hybridization Signal among the Different Histological Structures in Normal Liver and during Oval Cell Proliferation and Differentiation

	Normal liver		Oval cell differentiation model				
	Hepatocytes	Bile ducts	Hepatocytes	Oval cells	Bile ducts	Intestinal glands	Foci
HNF1 α	+	-	+	++	++	++	+++
HNF1 β	+	-	+	+++	++	++	+++
HNF3 γ	+	-	+	+++	+	++	+++
HNF4	+	-	++	+	-	+++*	++++
C/EBP	+	-	+	++	+	+++	+++
C/EBP β	+	-	+	++++‡	-	-	±
DBP	+	-	+	++	+	++	++

Intensity of the signal is indicated by +.

* Although most intestinal glands strongly expressed HNF4 a subpopulation of the glands displayed low expression of the factor.

‡ Few subpopulations of oval cells exist that express low levels of C/EBP β .

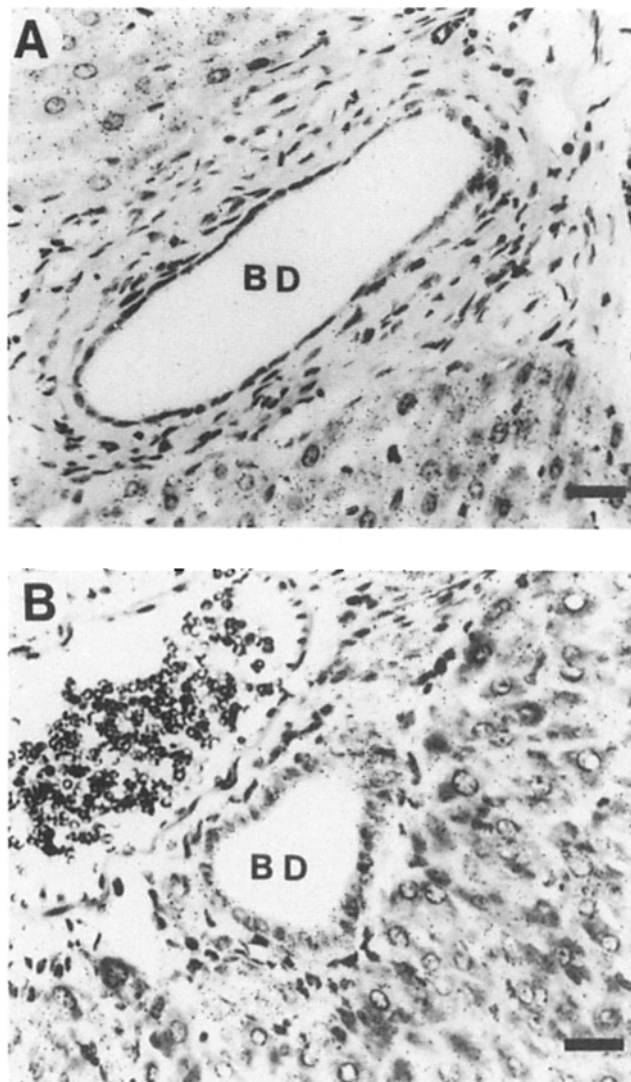


Figure 3. Expression of HNF1 α in control liver (A) and 4 d after partial hepatectomy (B). Note the high density of HNF1 α transcripts on the bile duct (BD) in B. Bar, 7 μ m.

When we examined the spatial expression of the transcription factors during the process of proliferation and differentiation of oval cells a very different picture of the cellular expression pattern was evident. For HNF1 α and HNF1 β a

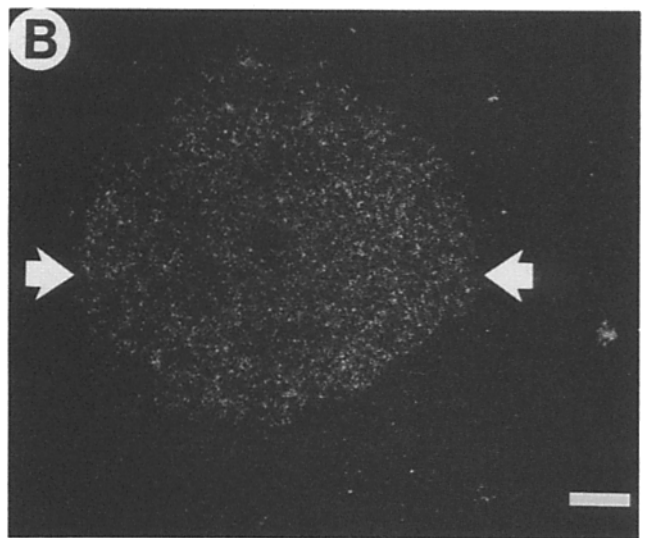
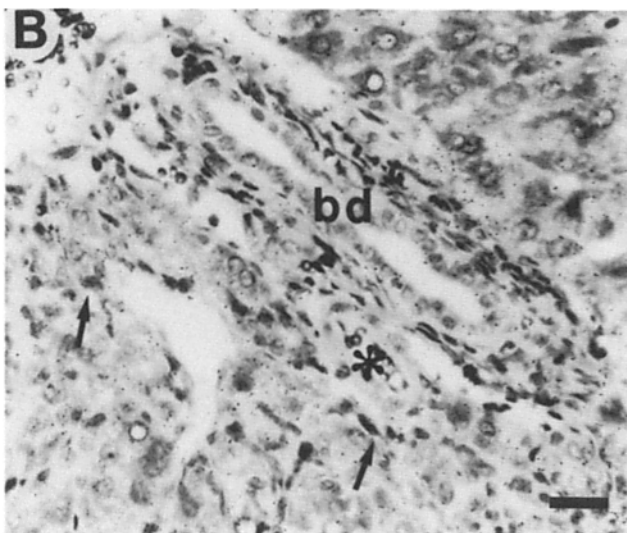
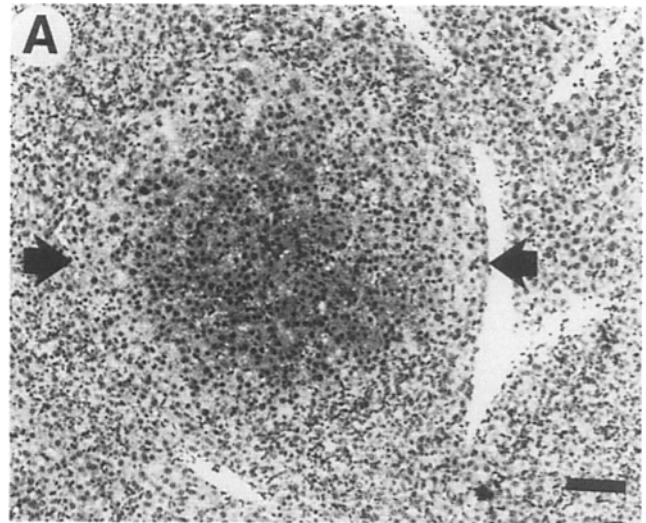
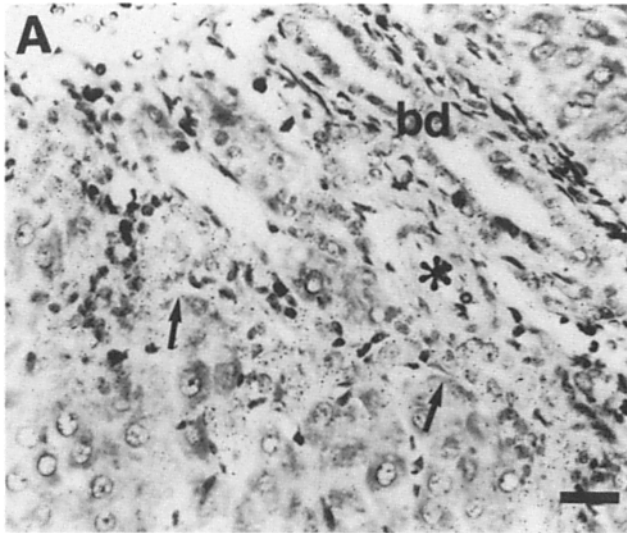


Figure 4. Expression of HNF1 β 4 days after partial hepatectomy (A). Note the expression in the bile duct (bd) and in the oval cells (arrows) while the hepatic artery (star) is negative. (B) Serial section hybridized with the sense probe. Bar, 7 μ m.

similar cellular distribution of transcripts was observed throughout the oval cell proliferation-differentiation process (Table I). The accumulation of silver grains was higher over the oval cells than the hepatocytes. The intestinal type glands were labeled equal to the oval cells, and interestingly the bile ducts became positive as well (Figs. 3 A and 4, A and B). However, the grain density was the highest over the “new” hepatocytes in the basophilic foci (Fig. 5, B and C).

For the HNF3 family in situ hybridization analysis was performed only with the HNF3 γ probe. The expression pattern of this transcription factor was similar to that of the HNF1 α and HNF1 β with slightly more grains accumulating in the oval cells, the bile ducts and the basophilic foci, than over the hepatocytes (Fig. 6, A, B, and C).

In contrast to all the other transcriptional factors studied, HNF4 transcripts increased in the hepatocytes and although there were grains over the oval cells, the level of accumula-

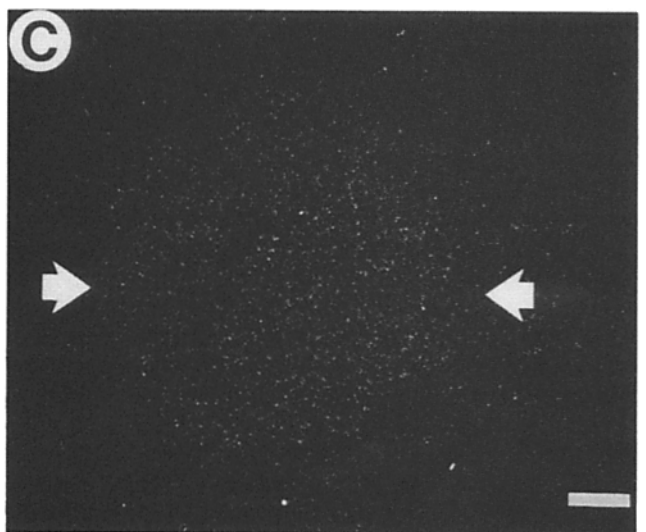


Figure 5. Expression of HNF1 α (B) and C/EBP (C) in a basophilic focus 8 d after partial hepatectomy in serial sections. (A) Bright field; (B and C) dark field illumination. Bar, 28 μ m.

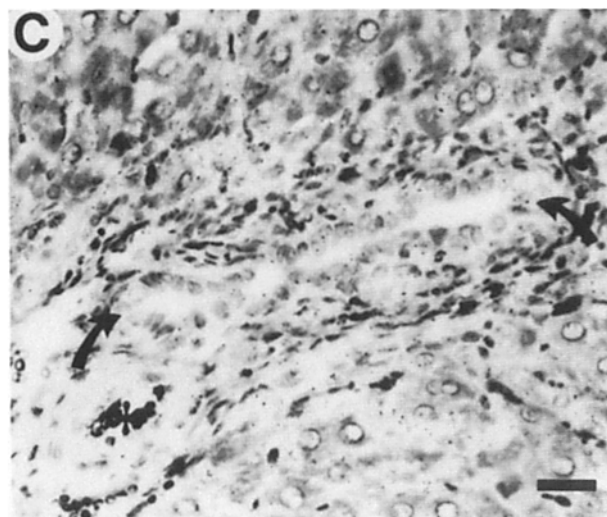
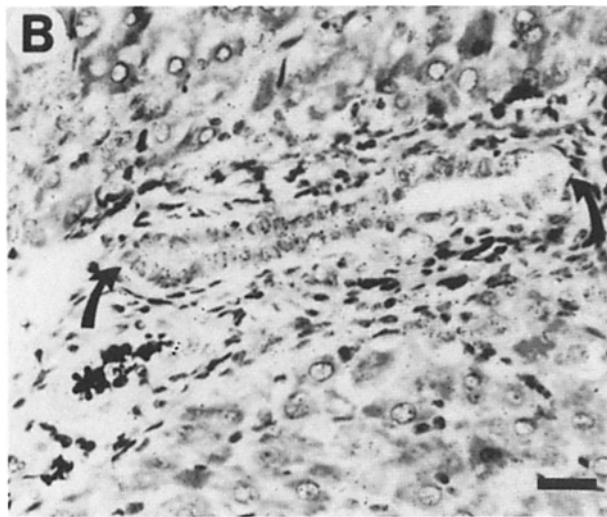
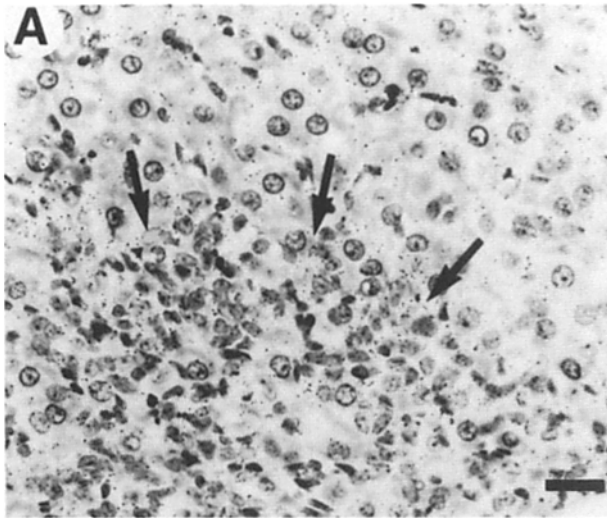


Figure 6. Expression of HNF3 γ 6 d after partial hepatectomy. Note the higher grain density over the oval cells (A) and the bile ducts (B) (curved arrows), and (C) serial section hybridized with sense probe. Bar, 7 μ m.

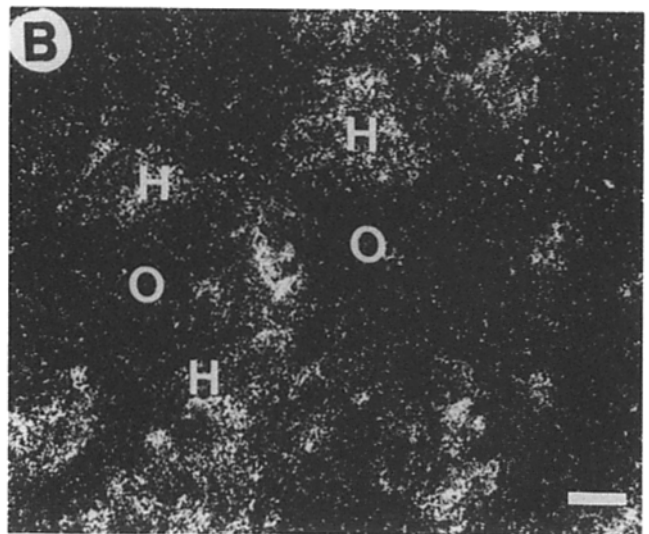
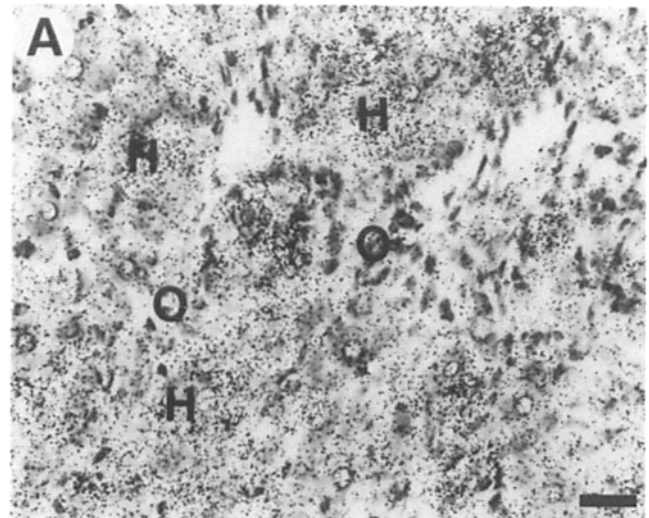


Figure 7. HNF4 expression 9 d after partial hepatectomy. Note higher density of grains on hepatocytes (H) than on oval cells (O). (A) Bright; (B) dark field illumination of the same field. Bar, 7 μ m.

tion was definitely lower (Fig. 7). Furthermore, the bile ducts remained negative. The grain density was the highest above the basophilic foci (Fig. 8, A and B). Some of the intestinal type glands were highly positive while others showed no accumulation of grains (Fig. 8, A and B).

Although the steady-state levels of C/EBP as revealed by the Northern blot analysis did not change significantly during the sequence of oval cell proliferation and differentiation (Fig. 2), the in situ hybridization showed that the distribution of the transcripts was not even among the different cell types. Again the expression was higher over the oval cells, intestinal type glands (data not shown) and basophilic foci (Fig. 5 C).

C/EBP β showed a unique distribution pattern. The Northern blot analysis (Fig. 2) showed an increased level of transcripts throughout the process of oval proliferation differentiation. The in situ hybridization revealed that this high steady-state level was caused by an exclusive increased accumulation of transcripts in the oval cells (Fig. 9, A and B).

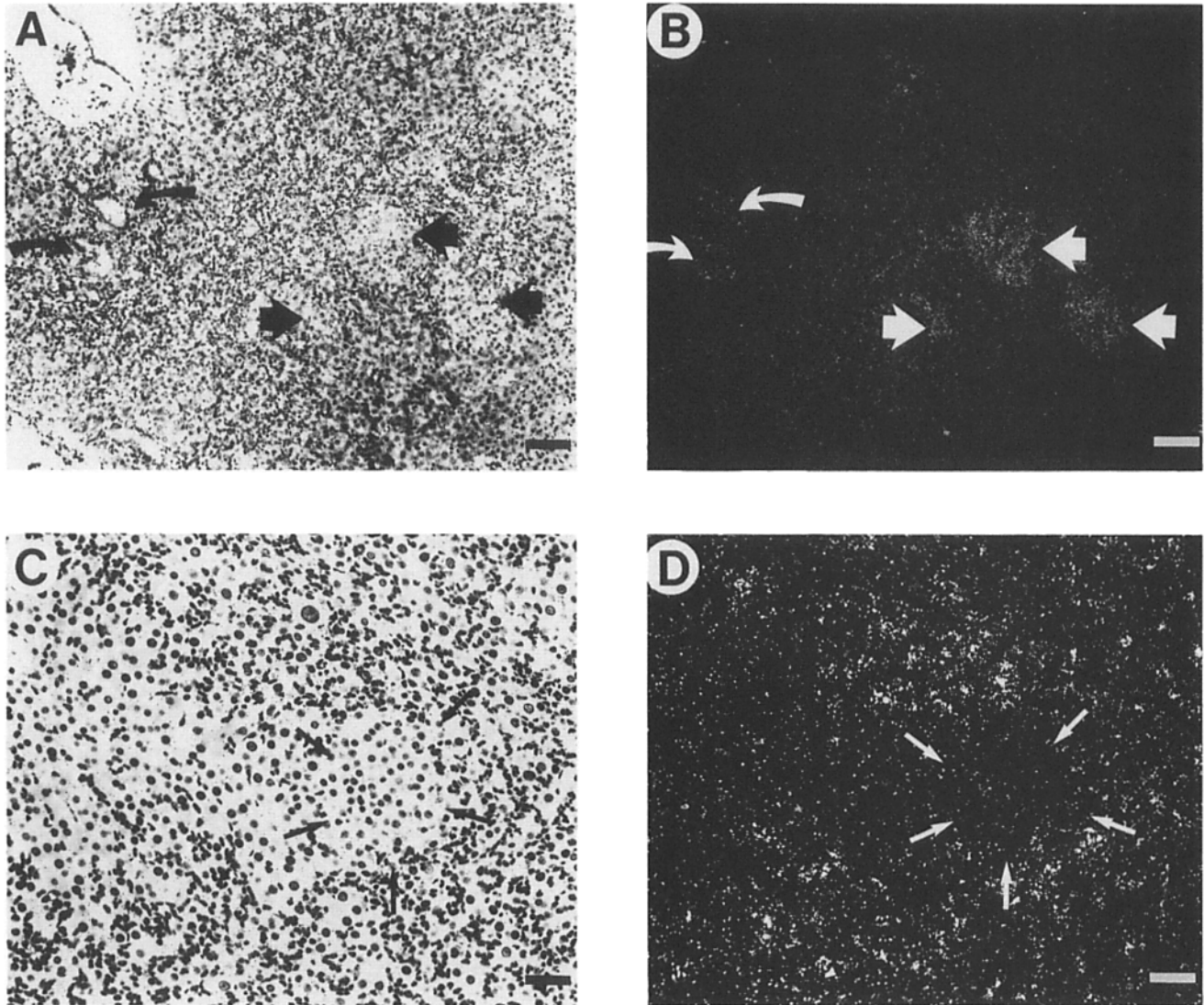


Figure 8. HNF4 expression 9 d after partial hepatectomy (*A* and *B*). Thick arrows show small but positive basophilic foci, curved arrows mark positive intestinal type glands. (*C* and *D*) C/EBP β expression 11 d after partial hepatectomy, a basophilic focus (*arrows*) lacks grains while some of the surrounding oval cells are positive. (*A* and *C*) Bright; (*B* and *D*) dark field illumination. Bars: (*A* and *B*) 28 μ m; (*C* and *D*) 14 μ m.

However, unlike the other factors only a small subpopulation of the oval cells was very heavily labeled, mostly the cells which showed ductular formation. The bile ducts (data not shown) and the intestinal type glands (Fig. 10, *A* and *B*) were negative for this probe as were the basophilic foci (Fig. 8, *C* and *D*). DBP transcripts accumulated to a higher extent in the oval cells than in the hepatocytes and the basophilic foci. Intestinal metaplastic glands expressed DBP about at the level of the oval cells (Fig. 10, *C* and *D*).

Discussion

At the 16th day of embryonic development the rat liver is mainly composed of relatively well-differentiated hepatoblasts. The hepatoblasts express high levels of AFP, produce albumin, but do not express tyrosine aminotransferase which is characteristic of mature adult hepatocytes (Shiojiri et al.,

1991). As liver development progresses, the liver structure starts to resemble that of the adult liver, ductal plates are being formed from the hepatoblasts surrounding the portal tracts and later the bile epithelium develops (Van Eyken et al., 1988). In the present study we have shown that the steady-state mRNA levels of four "liver enriched" transcription factors (HNF1 α , HNF3 α , HNF4, C/EBP β) gradually decrease during the late period of embryonic liver development while three factors (HNF1 β , HNF3 β , and DBP) increase. However, it is important to note that the hemopoietic cell compartment contributes significantly to the liver mass during both the fetal and perinatal period. The possibility therefore exists that these cells may contribute to the steady-state mRNA levels of the transcriptional factors. This possibility appears to be unlikely since earlier in situ hybridization studies on the expression of C/EBP and HNF1 in developing mouse liver showed that the transcripts for these factors were

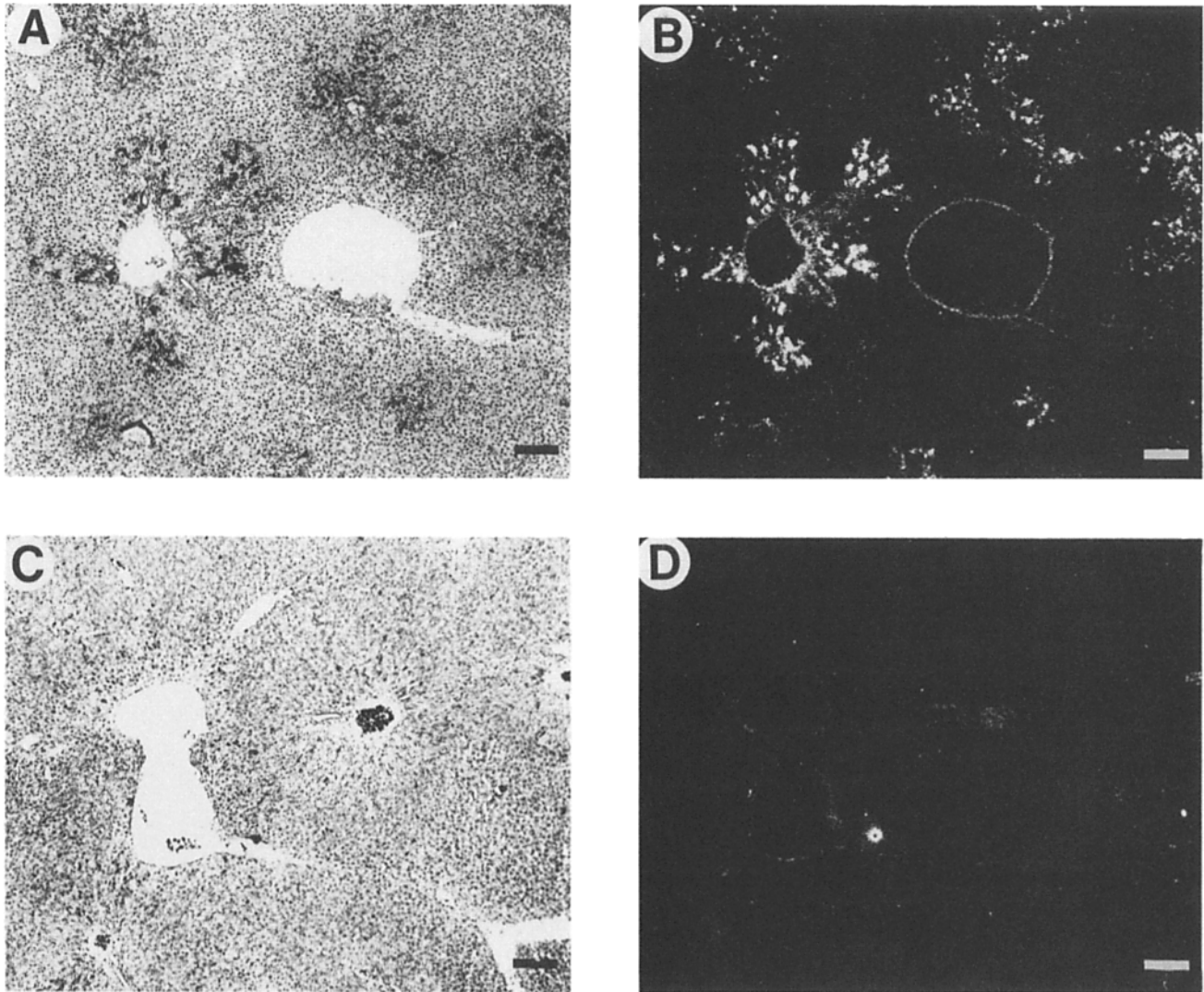


Figure 9. Serial sections hybridized with antisense (*A* and *B*) and sense (*C* and *D*) *C/EBPβ* probe. The oval cells, especially those showing ductal arrangement, are strongly positive for the antisense probe. The endothelial lining of the blood vessels shows some aspecific binding. (*A* and *C*) Bright; (*B* and *D*) dark field illumination. Bar, 56, μm .

only detected in liver epithelial cells (Kuo et al., 1990*b*; and Blumenfeld et al., 1991). Therefore, it appears that the gene expression pattern of the transcription factors gradually changes as the hepatoblasts mature to hepatocytes in late gestation and the perinatal period. This is, of course, in agreement with studies showing that AFP, albumin, and other genes are differentially regulated during development by particular combinations of the transcription factors examined in the present study (Van Ooij et al., 1992; Xanthopoulos et al., 1991; Herbst et al., 1991; Ott et al., 1991). The increasing mRNA levels observed for HNF1 β and decreasing levels for HNF1 α in late gestation and the perinatal period is surprising. HNF1 β has usually been associated with a less-differentiated phenotype (Mendel et al., 1991*a*; Baumhueter et al., 1988; Kuo et al., 1990*a*) and it has previously been shown that HNF1 β expression precedes that of HNF1 α during development in the mouse (Ott et al., 1991). The steady-state mRNA level decreased for factors (HNF1 α , -3, -4) classified as “establishment factors” by Lai and Dar-

nell (1991). These regulator proteins are probably more important determinants in liver development prior to day 16 (Griffo et al., 1993) while DBP, which is expressed only in the postnatal life, is called “maintenance factor” by Lai and Darnell (1991).

It has been proposed that oval cells which can differentiate into hepatocytes (Evarts et al., 1987*b*; Lemire et al., 1991) are derived from a compartment of facultative stem cells located in or around the small bile ducts in the adult rat liver (Aterman, 1992; Sell, 1993). Proliferating oval cells are composed of heterogeneous subpopulations (Fausto et al., 1987) of which most express albumin together with AFP and other markers of hepatoblasts. We were, therefore, interested in examining if we could observe changes in the expression of the specific transcription factors during the proliferation and differentiation of oval cells similar to those observed during normal liver development. As summarized in Table I, transcripts for all the transcription factors, with the exception of HNF4, were present at higher levels in the

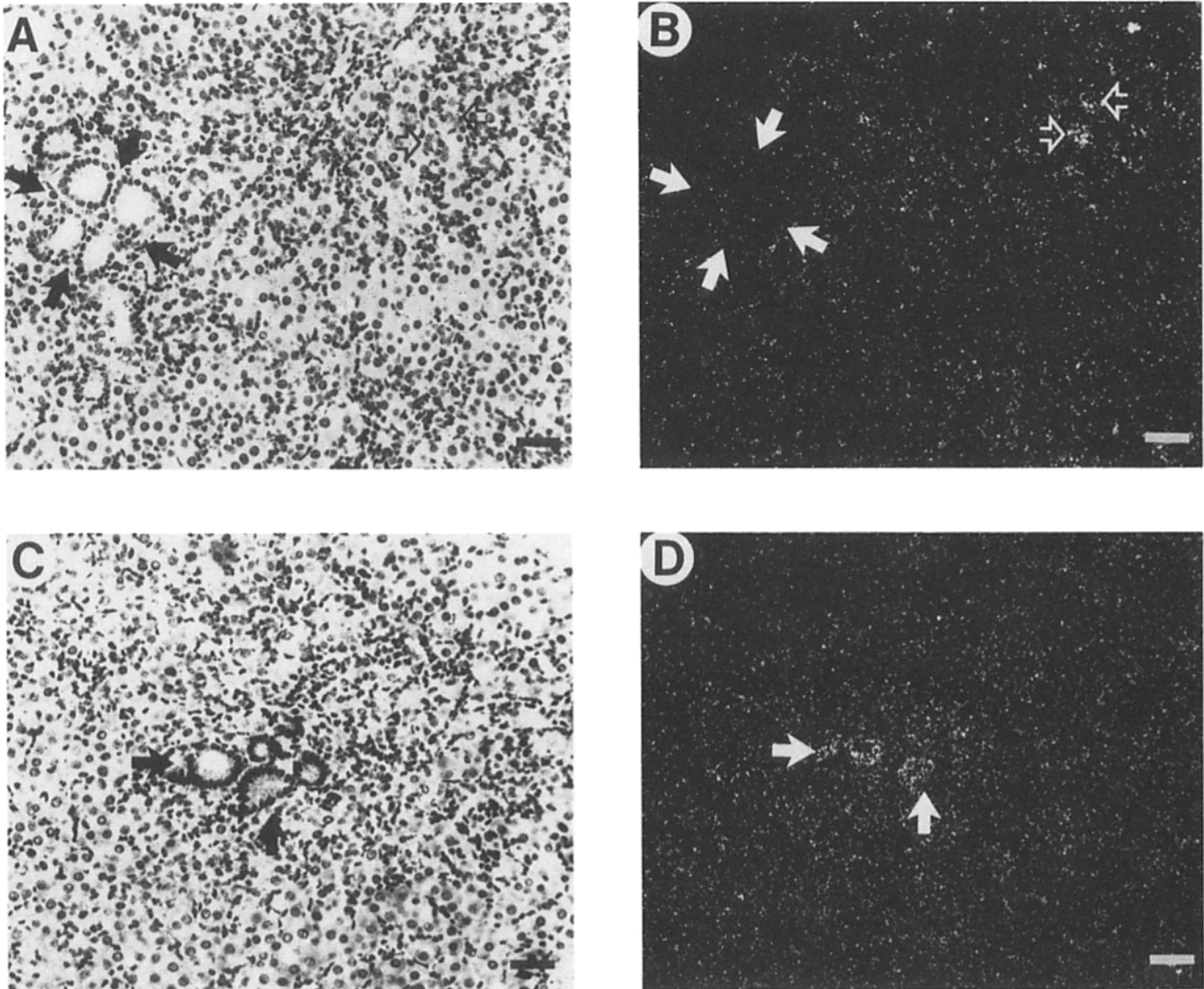


Figure 10. No C/EBP β transcripts are detected in intestinal type gland (*thick arrows*) 9 d after partial hepatectomy (*A and B*) while oval cells are positive (*open arrows*). Similar glands are positive for DBP 8 d after partial hepatectomy (*C and D*). (*A and B*) Bright field; (*C and D*) dark field illumination. Bar, 14 μ m.

oval cells than in the surrounding hepatocytes strongly supporting the proposed lineage affiliation to hepatoblasts. The level of transcript accumulation in individual oval cells appeared similar at the different time points after partial hepatectomy. Furthermore, these cells did not show any significant morphological alterations until they formed foci of basophilic hepatocytes. Therefore, it is likely that most of the oval cells were at a similar stage of differentiation during the early expansion of the oval cell compartment between the second and the ninth day after partial hepatectomy.

The formation of small foci of basophilic hepatocytes is a very critical step in the differentiation of oval cells. We hypothesize that this is the point at which a very small fraction of the oval cells becomes irreversibly committed to the hepatocytic lineage. The cells become morphologically similar to the hepatocytes, albumin expression sharply increases (Evarts et al., 1987a), AFP expression gradually decreases (Evarts et al., 1989; Bisgaard et al., 1994) and the production of proteins present only in highly differentiated

hepatocytes, e.g., P-glycoprotein (Nakatsukasa et al., 1992) is initiated. At present, the critical mechanism initiating these events is unknown. However, most of the “establishment transcriptional factors” including HNF1 α and HNF3 γ are upregulated in the oval cells when compared to the surrounding hepatocytes and high expression of these factors is maintained or even further increased in the foci of basophilic hepatocytes. Of special interest is the expression of HNF4. Recent reports by Kuo et al. (1992) and Tian and Schibler (1991) have led to the suggestion that expression of HNF4 may act upstream in a cascade activating other hepatocyte-specific factors. The sudden upregulation of HNF4 observed in the foci of basophilic hepatocytes as compared to the oval cells may be a trigger of further upregulation of the downstream factors involved in the differentiation of oval cells to hepatocytes and therefore may play a key role in the final, hepatocyte lineage commitment.

During embryonic development the bile ducts arise from hepatoblasts surrounding the portal tracts (Shiojiri et al.,

1991; Van Eyken et al., 1988). It is therefore noteworthy that we were unable to detect stable transcripts for any of the nine transcription factors examined in the bile ducts of the normal adult liver. However, all the factors with the exception of HNF4 and C/EBP β are expressed in the mature bile ducts and ductules at the earliest stages of oval cell proliferation. This is a striking observation suggesting that a large proportion of the biliary epithelial cells can respond to a combination of loss of liver mass and impaired regenerative capacity of existing hepatocytes by expressing the "establishment transcriptional factors." In this context it is important to note that available evidence suggests that oval cells are derived from the smaller branches, possibly the terminal bile ductules, of the bile duct tree (Evarts et al., 1993; Sell, 1993). It is, therefore, likely that components in addition to the transcriptional factors may determine the segment of the biliary ducts constituting the hepatic stem cell compartment. At present it is not clear what these other components are, but differential expression of growth factor/receptor systems and the capacity for extended proliferation may be significant determinants (Evarts et al., 1993). Thus, we hypothesized that expression of a selective combination of transcription factors, possibly HNF1 and -3 is necessary but not sufficient for the activation of the putative hepatocyte stem cell compartment.

In summary, we have examined the expression of nine "liver-enriched" transcription factors at the level of mRNA during the late stages of liver development and during differentiation of oval cells to hepatocytes in the adult liver. From the results we propose that the kinetics of differentiation of hepatoblast-like cells to mature hepatocytes may be significantly different in two models. During ontogenesis the primitive endodermal cells of the liver anlage probably mature to hepatocytes through a gradual process. This is reflected in the gradual changes of the transcriptional factors during embryonic development and the perinatal period. In contrast, the maturation of the liver stem cells to hepatocytes may happen in two discontinuous steps. The first step is the activation of the facultative stem cell compartment from which the oval cells arise as a distinct phenotype. The proliferating oval cells appear not to change phenotypically until the sudden upregulation of HNF4 occurs. We hypothesize that this second step may commit the oval cells irreversibly to the hepatocytic lineage. Other changes may result in the commitment of oval cells to bile epithelium. Finally, although the formation of intestinal type glands from oval cells may reflect the developmental plasticity of the oval cell compartment it could also illustrate a derailment of the regenerative process that can occur when the hepatic stem cell compartment is activated.

We are aware of the limitation in our studies. The regulation of transcriptional factors has been shown to occur at several different levels. There are examples of regulation occurring posttranscriptionally (Xanthopoulos et al., 1991), by protein phosphorylation (Wegner et al., 1929) as well, and by heterodimerization (Ron and Habener, 1992; Mendel et al., 1991a,b). An interesting example is C/EBP β . Descombes and Schibler (1991) have very elegantly demonstrated that two different proteins are translated from the same mRNA molecule using two translation start sites. These two DNA binding proteins, liver activating protein and liver inhibitor protein have directly opposite effects on

gene transcription when binding to the C/EBP consensus sequence of target genes. However, the expression of most of the known transcription factors in the liver is found to be regulated at the transcriptional level (Johnson, 1990; Cereghini et al., 1990; Tian and Schibler, 1991; Xanthopoulos et al., 1989; DeSimone and Cortese, 1992). Therefore we believe that measuring the steady-state mRNA levels of the liver enriched transcription factors particularly by in situ hybridization analysis have provided useful information about their general activity during the cellular events leading to hepatocyte commitment and differentiation.

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References

- Alberts, B., D. Bray, J. Lewis, M. Raff, R. K. Roberts, and T. O. Watson. 1989. *Molecular Biology of the Cell*, Garland Publishing Co. 553 pp.
- Aterman, K. 1992. The stem cells of the liver—a selective review. *J. Cancer Res. Clin. Oncol.* 118:87–115.
- Baumhueter, S., and G. R. Crabtree. 1988. A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the fibrogen gene promoter as HNF1. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2485–2493.
- Baumhueter, S., D. B. Mendel, P. B. Conley, C. J. Kuo, C. Turk, M. K. Graves, C. A. Edwards, G. Courtois, and G. R. Crabtree. 1990. HNF-1 shares three sequence motifs with the POU domain proteins and is identical to LF-B1 and APF. *Genes Dev.* 4:372–379.
- Birkenmeier, E. M., B. Gwynn, S. Howard, Y. Yerry, Y. T. Gordon, W. H. Landshulz, and S. L. McKnight. 1989. Tissue specific expression, developmental regulation and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev.* 3:1146–1156.
- Bisgaard, H. C., P. Nagy, P. T. Ton, Z. Hu, and S. S. Thorgeirsson. 1994. Modulation of keratin 14 and α -fetoprotein expression during hepatic oval cell proliferation and liver regeneration. *J. Cell. Physiol.* In press.
- Blumenfeld, M., M. Maury, T. Chouard, M. Yaniv, and H. Condamine. 1991. Hepatic nuclear factor 1 (HNF1) shows a wider distribution than products of its known target genes in developing mouse. *Development.* 113:589–599.
- Cereghini, S., M. Yaniv, and R. Cortese. 1990. Hepatocyte dedifferentiation and extinction is accompanied by a block in the synthesis of mRNA coding for the transcription factor HNF1/LFB1. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2257–2263.
- Clevidence, D. E., D. G. Overdier, W. Tao, X. Qian, L. Pani, E. Lai, and R. H. Costa. 1993. Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding-domain family. *Proc. Natl. Acad. Sci. USA.* 90:3948–3952.
- Courtois, G., S. Baumhueter, and G. R. Crabtree. 1988. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. *Proc. Natl. Acad. Sci. USA.* 85:7937–7941.
- Crabtree, G. R., U. Schibler, and M. P. Scott. 1992. Transcriptional regulatory mechanisms in liver and midgut morphogenesis of vertebrates and invertebrates. In *Transcriptional Regulation*. S. S. McKnight and K. R. Yamamoto, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1063–1102.
- Descombes, P., M. Chojkier, S. Lichsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* 4:1541–1551.
- Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell.* 67:569–579.
- De Simone, V., and R. Cortese. 1992. Transcription factors and liver-specific genes. *Biochim. Biophys. Acta.* 1132:119–126.
- DiPersio, C. M., D. A. Jackson, and K. S. Zaret. 1991. The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mol. Cell. Biol.* 11:4405–4414.
- Evarts, R. P., P. Nagy, E. Marsden, and S. S. Thorgeirsson. 1987a. A precursor-product relationship exists between oval cells and hepatocytes in rat liver. *Carcinogenesis.* 8:1737–1740.
- Evarts, R. P., P. Nagy, E. Marsden, and S. S. Thorgeirsson. 1987b. In situ hybridization studies on expression of albumin and α -fetoprotein during the early stage of neoplastic transformation in rat liver. *Cancer Res.* 47:5469–5475.
- Evarts, R. P., P. Nagy, H. Nakatsukasa, E. Marsden, and S. S. Thorgeirsson. 1989. In vivo differentiation of rat liver oval cells into hepatocytes. *Cancer Res.* 49:1541–1547.
- Evarts, R. P., H. Nakatsukasa, E. Marsden, C. C. Hsia, H. A. Dunsford, and S. S. Thorgeirsson. 1990. Cellular and molecular changes in the early stages of chemical hepatocarcinogenesis in the rat. *Cancer Res.* 50:3439–3444.

- Evarts, R. P., Z. Hu, K. Fujio, E. R. Marsden, and S. S. Thorgeirsson. 1993. Activation of hepatic stem cell compartment in the rat: role of transforming growth factor α , hepatocyte growth factor, and acidific fibroblast growth factor in early proliferation. *Cell Growth Differ.* 4:555-561.
- Farber, E. 1956. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.* 16:142-148.
- Fausto, N., N. L. Thompson, and L. Braun. 1987. Purification and culture of oval cells from rat liver. In *Cell Separation Methods and Selected Applications*. T. G. Pretlow and T. P. Pretlow, editors. Academic Press, Orlando, FL. 45-77.
- Frain, M., G. Swart, P. Monaci, A. Nicosia, S. Stämpfli, R. Frank, and R. Cortese. 1989. The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. *Cell.* 59:145-157.
- Fraslin, J.-M., B. Kneip, S. Vaulont, D. Glaise, A. Munnich, and C. Guguen-Guillouzo. 1985. Dependence of hepatocyte-specific gene expression on cell-cell interactions in primary culture. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2487-2491.
- Griffo, G., C. Hamon-Benais, P.-O. Angrand, M. Fox, L. West, O. Lecoq, S. Povey, D. Cassio, and M. Weiss. 1993. HNF4 and HNF1 as well as a panel of hepatic functions are extinguished and reexpressed in parallel in chromosomally reduced rat hepatoma-human fibroblast hybrids. *J. Cell Biol.* 121:887-898.
- Herbst, R. S., U. Nielsch, F. Sladek, E. Lai, L. E. Babiss, and J. E. Darnell, Jr. 1991. Differential regulation of hepatocyte-enriched transcription factors explains changes in albumin and transthyretin gene expression among hepatoma cells. *New Biol.* 3:289-296.
- Houart, C., J. Szpirer, and C. Szpirer. 1990. The α -fetoprotein proximal enhancer: localization, cell specificity and modulation of dexamethasone. *Nucleic Acids Res.* 18:6277-6282.
- Johnson, P. F. 1990. Transcriptional activators in hepatocytes. *Cell Growth Differ.* 1:47-51.
- Kimbrough, R. D., R. E. Linder, and T. B. Gaines. 1972. Morphological changes in liver of rats fed polychlorinated biphenyls. *Arch. Environ. Health.* 25:354-364.
- Kuo, C. F., K. G. Xanthopoulos, and Y. E. Darnell. 1990b. Fetal and adult localization of C/EBP: evidence for combinational action of transcription factors in cell specific gene expression. *Development.* 109:473-481.
- Kuo, C. J., P. B. Conley, L. Chen, F. M. Sladek, J. E. Darnell, Jr., and G. R. Crabtree. 1992. A transcriptional hierarchy involved in mammalian cell-type specification. *Nature (Lond.)*. 355:457-461.
- Kuo, C. J., P. B. Conley, C.-L. Hsieh, U. Francke, and G. R. Crabtree. 1990a. Molecular cloning, functional expression, and chromosomal localization of mouse hepatocyte nuclear factor 1. *Proc. Natl. Acad. Sci. USA.* 87:9838-9842.
- Lai, E., and J. E. Darnell, Jr. 1991. Transcriptional control in hepatocytes: a window on development. *Trends Biochem. Sci.* 16:427-430.
- Lai, E., V. R. Prezioso, E. Smith, O. Litvin, R. H. Costa, and J. E. Darnell, Jr. 1990. HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. *Genes Dev.* 4:1427-1436.
- Lai, E., V. R. Prezioso, W. Tao, W. S. Chen, and J. E. Darnell, Jr. 1991. Hepatocyte nuclear factor 3 α belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head*. *Genes Dev.* 5:416-427.
- Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* 2:786-800.
- Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science (Wash. DC).* 243:1681-1688.
- Lemire, J. M., N. Shiojiri, and N. Fausto. 1991. Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. *Am. J. Pathol.* 139:535-552.
- Lichsteiner, S., and U. Schibler. 1989. A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. *Cell.* 57:1179-1187.
- Liu, J.-K., C. M. DiPersio, and K. S. Zaret. 1991. Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro. *Mol. Cell. Biol.* 11:773-784.
- McKnight, S. L., and K. R. Yamamoto. 1992. *Transcriptional Regulation*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1335 pp.
- Mendel D. B., L. P. Hansen, M. K. Graves, P. B. Conley, and G. R. Crabtree. 1991a. HNF-1 α and HNF-1 β (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. *Genes Dev.* 5:1042-1056.
- Mendel, D. B., P. A. Khavari, P. B. Conley, M. K. Graves, L. P. Hansen, A. Admon, and G. R. Crabtree. 1991b. Characterization of a co-factor that regulates dimerization of a mammalian homeodomain protein. *Science (Wash. DC).* 254:1762-1767.
- Moorman, A. F., M. J. B. van den Hoff, P. A. J. de Boer, R. Charles, and W. H. Lamers. 1991. The dynamics of expression of C/EBP mRNA in the adult rat liver lobules qualifies it as a pericentral mRNA. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 288:133-137.
- Mueller, C. R., P. Maire, and U. Schibler. 1990. DBP, a liver-enriched transcriptional factor, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell.* 61:279-291.
- Nakabayashi, H., K. Watanabe, A. Saito, A. Otsuru, K. Sawadaishi, and T. Tamaoki. 1989. Transcriptional regulation of α -fetoprotein expression by dexamethasone in human hepatoma cells. *J. Biol. Chem.* 264:266-271.
- Nakatsukasa, H., R. P. Evarts, R. K. Burt, P. Nagy and S. S. Thorgeirsson. 1992. Cellular pattern of multidrug-resistance gene expression during chemical hepatocarcinogenesis in the rat. *Mol. Carcinog.* 6:190-198.
- Ott, M.-O., J. Rey-Camos, S. Cereghini, and M. Yaniv. 1991. vHNF1 is expressed in epithelial cells of distinct embryonic origin during development and precedes HNF1 expression. *Mech. Dev.* 36:47-58.
- Poli, V., F. P. Mancini, and R. Cortese. 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell.* 63:643-653.
- Rao, M. S., R. D. Bendayan, R. D. Kimbrough, and J. K. Reddy. 1986. Characterization of pancreatic-type tissue in the liver of rats induced by polychlorinated biphenyls. *J. Histochem. Cytochem.* 34:197-201.
- Ron, D., and J. F. Habener. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* 6:439-453.
- Schweizer, G., and K. Goertler. 1980. Synthesis in vitro of keratin polypeptides directed by mRNA isolated from newborn and adult mouse epidermis. *Eur. J. Biochem.* 112:243-249.
- Sell, S. 1993. The role of determined stem-cells in the cellular lineage of hepatocellular carcinoma. *Int. J. Dev. Biol.* 37:189-201.
- Shiojiri, N., J. M. Lemire, and N. Fausto. 1991. Cell lineages and oval cell progenitors in rat liver development. *Cancer Res.* 51:2611-2620.
- Sigal, S. H., S. Brill, A. S. Fiorino, and L. M. Reid. 1992. The rat as a stem cell and lineage system. *Am. J. Physiol.* 263:G139-G148.
- Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell, Jr. 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* 4:2353-2365.
- Tatematsu, M., K. Thohru, A. Medline, and E. Farber. 1985. Intestinal metaplasia as a common option of oval cells in relation to cholangiofibrosis in livers of rats exposed to 2-acetylaminofluorene. *Lab. Invest.* 52:354-362.
- Thorgeirsson, S. S., and R. P. Evarts. 1990. Hepatic stem cell compartment in the rat. In *Hepatic Metabolism and Disposition of Endo- and Xenobiotics*. K. W. Bock, S. Matern, W. Gerok, and R. Schmidt, editors. Falk Symposium, Kluwer Academic Publishers, Dordrecht/Boston/London. 57:19-26.
- Tian, J.-M., and U. Schibler. 1991. Tissue-specific expression of the gene encoding hepatocyte nuclear factor 1 may involve hepatocyte nuclear factor 4. *Genes Dev.* 5:2225-2234.
- Tripodi, M., C. Abbott, N. Vivian, R. Cortese, and R. Lovell-Badge. 1991. Disruption of the LF-A1 and LF-B1 binding sites in the human alpha-1-antitrypsin gene has a differential effect during development in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3177-3182.
- Van Eyken, P., R. Scot, and V. Desmet. 1988. Intrahepatic bile duct development in the rat: a cyokeratin-immunohistochemical study. *Lab. Invest.* 59:52-59.
- van Ooij, C., R. C. Snyder, B. W. Paeper, and G. Duester. 1992. Temporal expression of the human alcohol dehydrogenase gene family during liver development correlates with differential promoter activation by hepatocyte nuclear factor 1, CCAAT/enhancer-binding protein α , liver activator protein, and D-element-binding protein. *Mol. Cell. Biol.* 12:3023-3031.
- Wegner, M., Z. Cao, and M. G. Rosenfeld. 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBP β . *Science (Wash. DC).* 256:370-373.
- Williams, S. C., C. A. Cantwell, and P. F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev.* 5:1553-1567.
- Wright, N. A., R. Poulson, G. W. M. Stamp, P. A. Hall, R. E. Jeffery, G. M. Longcroft, M. Rio, G. Tomasetto, and P. Chambooy. 1991. EGF/URO induces expression of regulatory peptides in damaged human gastrointestinal tissues. *J. Pathol.* 162:279-284.
- Wuarin, J., E. Falvey, D. Lavery, D. Talbot, E. Schmidt, V. Ossipow, P. Fajallaz, and U. Schibler. 1992. The role of the transcriptional activator protein DBP in circadian liver gene expression. *J. Cell Sci.* 16:123-127.
- Xanthopoulos, K. G., J. Mirkovitch, T. Decker, C. F. Kuo, and J. E. Darnell, Jr. 1989. Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP. *Proc. Natl. Acad. Sci. USA.* 86:4117-4121.
- Xanthopoulos, K. G., V. R. Prezioso, W. S. Chen, F. M. Sladek, R. Cortese, and J. E. Darnell, Jr. 1991. The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3 and HNF-4, protein factors that govern liver-specific transcription. *Proc. Natl. Acad. Sci. USA.* 88:3807-3811.