Cellular Analysis by In Situ Hybridization and Immunoperoxidase of Alpha-Fetoprotein and Albumin Gene Expression in Rat Liver during the Perinatal Period

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Abstract. To analyze at the cellular level the decrease in alpha-fetoprotein (AFP) gene expression during the early postnatal growth, we searched for AFP gene transcripts by in situ hybridization using a specific cDNA probe, and for the corresponding protein by immunocytochemistry, on rat liver sections at various times of the perinatal period. The relative number of mRNA sequences was evaluated by Northern blot analysis. Albumin (ALB) gene expression was studied simultaneously with the same techniques.

In 17-19-d-old fetuses all hepatocytes express simultaneously, for both genes, the mRNAs and the corresponding proteins. During the first postnatal weeks, at a time when the global number of AFP mRNA molecules decreases, all hepatocytes still contain cytoplasmic transcripts and protein. A zonal heterogeneity in the level of AFP gene expression develops around the first week, a higher number of gene products being detected in perivenous than in periportal hepatocytes. This heterogeneity persists until the fourth week when AFP mRNA sequences and protein are barely detectable. All hepatocytes express the ALB gene after birth, but at around the second week, a periportal intensification of the in situ hybridization signal and immunostaining becomes apparent. Our data indicate that (a) co-expression of the AFP and ALB genes by all hepatocytes is a normal step in liver ontogeny; (b) the diminution of AFP gene expression after birth is not the result of the disappearance of specialized cell clones; and (c) zonal quantitative differences in the level of AFP and ALB gene expression are observed within the maturing liver lobule.

The synthesis of alpha-fetoprotein (AFP) and albumin (ALB), two major plasma proteins, undergoes sequential changes during mammalian embryonic and early postnatal development. AFP is the main plasma protein synthesized by the fetal liver and yolk sac. Its serum concentration falls very rapidly after birth to reach low, barely detectable levels in the adult individual (1, 12, 24, 28). In contrast, ALB is the dominant plasma protein synthesized by the adult liver and its serum concentration increases from low levels early in fetal development to high levels after birth and through adult life.

Recent molecular analyses have clearly demonstrated that the differential expression of the AFP and ALB gene during liver development is mainly regulated at the transcriptional level (21, 25, 31). Accumulation of both AFP and ALB mRNA molecules has been observed during the late gestational period. It is followed by a selective decrease in AFP mRNA transcription after birth (30, 31). However, the precise mechanisms responsible for the progressive transcriptional inactivation of the AFP gene at the cellular level remain obscure. More specifically, the question of whether this process, as reflected in the mRNA molecule number and in serum concentration, takes place synchronously in all hepatocytes or whether it occurs in a heterogeneous fashion by the disappearance of fetal cell clones, has not yet been unequivocally answered. In the same line, in situ cellular analysis of ALB gene transcription during the perinatal period has not been documented. Immunocytochemical techniques have been used to analyze the cellular localization of AFP and ALB in the course of liver development. They lead to conflicting results, in particular as concerns the percentage of hepatocytes engaged in AFP expression during the perinatal period (2, 8, 10, 14, 18, 22). This controversy could be explained by the differences in fixation and antibody permeabilization procedures, which have been shown to be critical in these techniques (6, 23).

At the present time, the most direct way to address these...
questions is to reveal the cellular AFP and ALB mRNA sequences on liver sections taken at different stages of rat liver development by an in situ hybridization analysis using specific cDNA probes. We report here the results of such an analysis. In addition, the number of specific AFP and ALB mRNA molecules present in the liver was evaluated using the Northern blot technique and, lastly, on parallel sections, we revealed the proteins by an immunoperoxidase technique. We show that the AFP and ALB genes are expressed simultaneously by all hepatocytes between the end of the gestation period and the fourth postnatal week. Therefore the decrease in AFP gene expression is not secondary to the disappearance of specialized AFP-producing clones. Finally, when the liver structure has matured into definitive liver cell plates, quantitative differences in transcriptional levels of both AFP and ALB genes take place within the hepatic lobule.

**Materials and Methods**

**Animals**

Animals of the Wistar strain (Charles River Breeding Laboratories, Inc., Saint-Aubin-Lez-Elbeuf, France) were used in all experiments. Fetuses were obtained from pregnant rats from days 17 to 21 of gestation. 1-5 wk-old neonates and adult rats were also examined. For each age tested, at least three animals from different litters were examined.

**RNA Preparations**

Total RNA was extracted directly from frozen tissue with guanidium thiocyanate essentially as described (9) using centrifugation through a dense cushion of cesium chloride to separate the RNA from the guanidium thiocyanate homogenate.

**Molecular Probes**

The ALB and AFP cDNA probes used in this study were the recombinant plasmids pRSA 13, pRSA 57, and pRSA 8, as well as pRAFP 65 and pRAFP 87 previously described (26, 27). For the in situ hybridization experiments, we used the ALB and AFP fragments, respectively, excised from the recombinant plasmids pRSA 8 and pRAFP 65. As a negative control, we used a 0.9-kb fragment isolated from pBR 325 digested by Alu I (Boehringer).

**Northern Blot Analysis of AFP and ALB mRNA Sequences**

Total RNA samples were fractionated electrophoretically using a denaturing formaldehyde/agarose gel system as described (26). The RNA was then transferred to BioDyne paper (Pall Corp., Glen Cove, NY). After baking at 80°C for 1 h, filters were prehybridized, hybridized, and washed essentially as described by the manufacturer. The filters were then autoradiographed as reported (11).

**Fixation Procedure**

For in situ hybridization, fetuses were immediately perfused for 5 min through the left heart ventricle with a 4% freshly prepared solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Livers from 1-5 wk-old neonates and from adult animals were processed as previously described (6). In all cases, perfusion fixation was followed by a 1-h immersion postfixation of liver slices in the same fixative and by extensive washings (48-72 h) in 0.1 M phosphate buffer (pH 7.4). Cryostat sections (6-8 μm) were collected on pretreated slides (see below), and stored at -20°C. They were analyzed within 1 mo.

**In Situ Hybridization**

(a) **Preparation of Tissue Sections.** Sections and coverslips were prepared essentially as described by Bernau et al. (6). The pretreatment of tissue samples was based on the procedures of Braglia et al. (7) and has been reported elsewhere (6). Briefly, slides were passed through graded ethanol, immersed in HCl 0.2 N for 20 min. After 30 min in 2× SSC buffer (1× SSC = 0.15 M NaCl, 0.015 M Na citrate) at 70°C, slides were incubated for 30 min at room temperature in a pronase (Calbiochem-Behring Corp., La Jolla, CA) solution at 0.25 mg/ml in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and immersed in 2 mg/ml glycine in PBS for 30 s. The slides were then postfixed in 4% paraformaldehyde for 20 min and dehydrated through graded ethanol. Control sections were treated with 1 mg/ml RNase A (Sigma Chemical Co., St. Louis, MO) in PBS for 1 h at 37°C before hybridization with a specific probe.

(b) **Hybridization of Labeled Probes to Tissue Sections.** After nick-translation, the labeled probes were ethanol precipitated after the addition of 500 μg sonicated salmon sperm DNA per μg of probe. The probes were then resuspended at a final concentration of 1 μg/ml in 50% deionized formamide (E. Merck, Darmstadt, Federal Republic of Germany), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution (0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 500 μg/ml yeast tRNA, 10% dextran sulfate (Pharmacia Biotech), Uppsala, Sweden). When 35S-labeled probes were used, 1 mM final dithiothreitol was added to the hybridization medium. In all experiments, controls included in situ hybridization with the labeled pBR 325 fragment and a pretreatment with RNase A before hybridization with a specific probe. The hybridization and washing procedures were essentially as described (6).

The slides were then air dried and immersed in NTB-2 (Eastman Kodak Co., Rochester, NY) emulsion diluted 1:1 with 0.6 M ammonium acetate at 45°C. The slides were developed in Kodak D99 and stained with 2% Giemsa in 0.01 M phosphate buffer (pH 7.4) for 30 min. Slides hybridized with 3H-labeled probes were exposed for 21 to 40 d; when hybridized with 35S-labeled probes, the exposure time was reduced to 4-12 d.

(c) **Quantitative Estimation of Lobular AFP and ALB mRNA Distribution.** A semi-automated estimation of the grain density over periporal and perivenous areas was performed using a Quantimet 720 image analyzer (Cambridge Instruments, Cambridge, England) and a PDF II/34 minicomputer (Digital Equipment Corporation, Galway, Ireland) (4). For a given slide exhibiting a zonal heterogeneity for AFP or ALB, 30 square fields of 3,364 μm² surrounding four portal spaces chosen at random and 30 square fields of the same surface surrounding four centrilobular veins, also chosen at random, were analyzed. After correction for the background estimated by the grain density over the same surface area of sinusoidal vascular spaces, the average number of grains per periporal or perivenous field was evaluated.

This method does not allow an overall estimation of grain density but does provide absolute values of the number of grains since the power of resolution of the analyzer is not sufficient to discriminate between clustered silver grains.

**Immunoperoxidase Technique**

Liver cryostat sections adjacent to those used for in situ hybridization were processed for detection of ALB and AFP as previously described (6). Briefly, after pronase treatment the sections were incubated with the following antibodies: (a) peroxidase (PO)-labeled anti-rat ALB prepared as described (23); (b) goat anti-rat AFP (25) followed by PO-labeled anti-goat IgGs (Institut Pasteur, Paris, France). Control reactions included, for ALB detection, incubation in PO-labeled anti-rat Ig (kindly provided by Dr. P. Druet) and for AFP, omission of the first antiserum. In all cases, before incubation with the antibodies, endogenous PO was inhibited by immersing the sections for 30 min at room temperature in methanol with 0.5% of hydrogen peroxide.

**Results**

**Molecular Analysis**

To evaluate the relative quantities of AFP and ALB molecules in the liver during the perinatal period, total RNA sam-

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Figure 1. Localization of AFP and ALB mRNAs and protein in 18-d-old embryo liver sections. (a and b) Sections hybridized with an AFP (a) or ALB (b) 3H-labeled cDNA probe, and exposed for 30 d. Numerous silver grains detecting the presence of cellular mRNAs are observed over all hepatocytes, but not to any appreciable extent over hematopoietic cells (arrows). (c) Control section preincubated with RNAse before hybridization. No significant hybridization signal is visible. The dark appearance of the hepatocyte cytoplasms is due to the Giemsa blue staining. (d and e) Adjacent sections immunostained for AFP (d) and ALB (e). Almost all hepatocytes contain cytoplasmic dark granular deposits detecting the presence of the proteins. Hematopoietic cells are negative (arrows). (f) Control section immunostained for Ig. All cells are negative. Bars, 20 μm.

Cellular Analysis by In Situ Hybridization and Immunoperoxidase

(a) In Fetal Liver (17th to 19th Day of Gestation). In situ hybridization was performed on liver sections of 17-19-d-old fetuses with an AFP or ALB 3H-labeled probe. In both cases, as shown in Fig. 1, a and b, essentially all hepatocytes contained numerous silver grains corresponding to mRNA-cDNA hybrids, mainly located over the cytoplasm. Signal intensity appeared homogeneously distributed, although slight variations in grain number were visible from one cell to another. In contrast, only a few nonspecific silver grains were observed on the hematopoietic foci (Fig. 1, a and b). In all experiments, the specificity of detection was assessed by observation of a dramatic decrease in the number of grains after treatment of the sections with RNase A, the few remaining grains being interpreted as background labeling (Fig. 1 c). Immunoperoxidase localization of AFP and ALB by incubation of liver sections with anti-rat AFP or ALB antibodies paralleled the results of in situ hybridization: both proteins were detected in essentially all hepatocytes as evidenced by the presence of dark brown granular deposits in the cytoplasm of the cells (Fig. 1, d and e). Control reactions using anti-rat Ig were always negative (Fig. 1 f).

(b) During Postnatal Development. In 1- and 5-d-old rat liver sections hybridized in situ with the AFP or ALB probe, all hepatocytes contained cytoplasmic and, to a lesser extent, nuclear mRNA-cDNA hybrids (Fig. 2 a). At both ages, no obvious difference in signal intensity was detectable between
the three zones of the hepatic lobule for either probe. Similarly, in 1- and 5-d-old rat liver sections, the immunoperoxidase analysis revealed the presence of AFP and ALB in all hepatocytes with an apparently homogeneous staining intensity within the hepatic lobule (Fig. 2 b).

In 1- and 2-wk-old rat liver sections, the \(^{3}H\)-labeled AFP probe still revealed numerous mRNA–cDNA hybrids in all hepatocytes. However, a heterogeneous signal intensity was observed between the different regions of the lobule: the hepatocytes surrounding the centrilobular veins were more intensively labeled than those around the portal zones. This signal heterogeneity was made more obvious upon using a probe labeled with \(^{35}S\)-labeled nucleotide precursors of higher specific activity than the \(^{3}H\)-labeled ones (Fig. 3, a and b). Adjacent sections hybridized with the \(^{35}S\)-labeled pBR fragment did not display a significant hybridization sig-

\[Figure 2. \] Localization of AFP mRNAs and protein in 1-d-old rat liver sections. CLV, centrilobular vein; PT, portal tract. (a) 1-d-old rat liver section hybridized with a \(^{35}S\)-labeled cDNA probe, and exposed for 8 d. All hepatocytes contain numerous cytoplasmic silver grains revealing AFP mRNAs; they are homogeneously distributed throughout the liver lobule. Note the absence of significant hybridization signal over the small dark hematopoietic foci still scattered in the sinusoids. (b) Adjacent liver section immunostained for AFP. All hepatocytes contain dark granular deposits which detect the presence of AFP. No difference in staining intensity is apparent within the liver lobule. Bars, 30 \(\mu\)m.

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Figure 3. Localization of AFP mRNAs and protein in 1-wk-old rat liver sections. CLV, centrilobular vein; PT, portal tract. (a) Section hybridized with a 35S-labeled cDNA probe, and exposed for 8 d. Silver grains detecting the presence of AFP mRNAs are present over all hepatocytes, but predominate over the perivenous area. Bar, 50 μm. (b) High magnification of the rectangular area delimited in a: the density of silver grains is higher on perivenous hepatocytes than on periportal hepatocytes. Bar, 30 μm.

(nal (Fig. 4). A quantitative estimation of grain density confirmed the presence of a higher number of silver grains on the perivenous than on the periportal areas (Table I). Immunoperoxidase localization revealed AFP in all hepatocytes, with zonal differences similar to those observed for the mRNA signal (Fig. 5).
Around the third week, scattered perivenous hepatocytes exhibited a strong hybridization signal for AFP mRNA sequences (Fig. 6a) while the great majority of the remaining hepatocytes presented a low but significant signal. As concerns the protein, it was only detected in a few hepatocytes exclusively located around the centrilobular veins (Fig. 6b). Finally, from the fourth week after birth onwards, in situ hybridization results were similar to those observed in adult liver: no significant cytoplasmic signal over the background was observed in the conditions used in this work (not shown) (6); in the same way, no further AFP-positive cells could be detected.

During the first two weeks of postnatal development, liver sections hybridized with the 3H- or 35S-labeled ALB probe or incubated with PO-labeled anti-rat ALB antibodies displayed a homogeneous hybridization signal and immunoperoxidase staining in all hepatocytes. Around the second week, the ALB hybridization signal became more intense in the periportal and mediolobular zones and remained as such in adult liver sections (Fig. 7a); a quantitative estimation of grain density confirmed the presence of a higher number of silver grains on the periportal than on the perivenous areas (Table II). A heterogeneous pattern of staining was also recorded for the protein with the immunoperoxidase technique (Fig. 7b).

**Discussion**

In this study, we demonstrate that the great majority of the fetal and newborn hepatocytes are actively engaged in both AFP and ALB gene expression during the perinatal period. Moreover we show that, for both genes, quantitative differences in the steady-state level of mRNA molecules develop within the liver lobule during the first postnatal weeks.

These results have been obtained in parallel by two independent techniques: in situ hybridization with specific AFP and ALB cDNA probes applied on liver sections at different stages of pre- and postnatal development, and immunoperoxidase localization of the corresponding proteins on parallel sections, with AFP- or ALB-specific antibodies. The nature and specificity of detection of the AFP and ALB mRNA sequences were ascertained by the observation of an important decrease in the number of grains corresponding to mRNA-cDNA hybrids in the hepatocytes after treatment.
with RNase A and, by the absence of a significant signal after hybridization with a pBR 325 fragment of approximately the same size as our probes. In addition, the absence of a significant number of autoradiographic grains on hematopoietic cells, which do not synthesize AFP or ALB, leads to the same conclusion (8, 18). The specificity of protein detection was controlled by incubation of liver sections with nonspecific sera and/or anti-Ig antibodies.

It has been proposed that the young mammalian liver is made of a cell mosaic consisting of functionally heterogeneous hepatocytes, some producing AFP, others ALB (2, 14, 18, 33). The results of our study show unambiguously that all hepatocytes simultaneously express the AFP and ALB genes, during the perinatal period comprised between day 17 of gestation and the third postnatal week. This observation confirms previous studies merely performed with immunocytochemical techniques in rat and pig (8, 10, 22). These data thus demonstrate that the capacity of all hepatocytes to co-express the AFP and ALB genes is a normal step in liver ontogeny.

The dramatic decrease in AFP gene expression that takes place during the early postnatal weeks is well demonstrated with Northern and dot blot analysis by a progressive decline in the number of AFP mRNA molecules from birth until the fourth week of life (21, 25; this study). At the cellular level, during this period, AFP transcripts are still detected in essentially all hepatocytes up to the fourth week after birth when adult levels of AFP gene expression are reached. Our data rule out the possibility that the cessation of AFP gene expression is due to the disappearance of specialized cell clones as suggested previously (18, 33), and provide strong evidence for a progressive shift of all hepatocytes from AFP/ALB- to ALB-producing phenotype proposed by other authors (3, 8, 24).

For both the AFP and ALB genes, a zonal heterogeneity in the level of expression appears to develop during the first weeks of postnatal life. It is not clear at the present time whether the different amounts of mRNA molecules detected in the perportal and perivenous hepatocytes reflect variations in transcription rate and/or in mRNA stability in these areas. With respect to ALB, a preferential perportal localization of both in situ hybridization signal and immunoperoxidase staining becomes apparent around the second week of life, and persists in the adult liver. Such a zonal heterogeneity has not been reported in our previous analysis of ALB gene expression in adult rat liver (6). These discrepancies could be accounted for by longer exposure times of the hybridized sections during the autoradiographic procedure in the present study, and the use of $^{35}$S-labeled probes of higher sensitivity. An intensification of the immunoperoxidase staining for ALB in the perportal zones had already been observed but not commented on by one of us (23). The cellular mechanisms responsible for the modulation of ALB gene expression within the liver lobule are unknown. Since ALB synthesis could be subjected to complex hormonal influence (17), quantitative differences in ALB gene expression might be related to hormonal factors acting differentially on the liver lobule zones. As regards AFP, a different zonal heterogeneity in the level of gene expression is

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Table II. Quantitative Estimation of the Density of Silver Grains Detecting ALB mRNA Sequences in the Hepatic Lobule of Four Different Rats

<table>
<thead>
<tr>
<th>Rat age</th>
<th>Number of grains per periporal field*</th>
<th>Number of grains per perivenous field*</th>
<th>Ratio perivenous/periporal fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 d</td>
<td>629.8 ± 80.74</td>
<td>517.6 ± 40.8</td>
<td>0.82</td>
</tr>
<tr>
<td>15 d</td>
<td>640.2 ± 35.2</td>
<td>583.6 ± 45.5</td>
<td>0.90</td>
</tr>
<tr>
<td>20 d</td>
<td>774.1 ± 38.8</td>
<td>699.0 ± 32.2</td>
<td>0.90</td>
</tr>
<tr>
<td>Adult</td>
<td>504.5 ± 64.2</td>
<td>458.2 ± 52.3</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* 30 fields of 3,364 μm², after correction for background.
† Mean ± SEM.
Figure 6. Localization of AFP mRNAs (a) and protein (b) in 3-wk-old rat liver sections. CLV, centrilobular vein. (a) Section hybridized with an AFP 35S-labeled cDNA probe, and exposed for 8 d. Scattered isolated hepatocytes around the centrilobular vein (arrows) contain a high number of silver grains detecting the presence of AFP mRNAs. The remaining hepatocytes contain a small, but significant, number of silver grains (compare with the number of silver grains obtained with a 35S-labeled pBR fragment synthesized at the same time, on Fig. 4). Bar, 20 µm. (b) Adjacent section immunostained for AFP. The dark granular deposits detecting the presence of AFP are observed in a few scattered perivenous hepatocytes. Bar, 30 µm.

Recorded. On 8-d-old rat liver sections, perivenous hepatocytes contain more AFP transcripts than hepatocytes located in the other zones of the liver lobule. This heterogeneous lobular level of AFP gene expression persists until the fourth week of life when adult levels of AFP gene expression are reached. As concerns the protein, until the third postnatal week, we observe a situation similar to the one for the mRNA sequences: a zonal heterogeneity with the perivenous zones more intensely stained than the other lobule areas. Similar observations have been made repeatedly in immunocytochemical studies (8, 10, 13, 22). At 3 wk, we record a discrepancy between the data obtained by in situ hybridization and immunoperoxidase. Although the majority of the hepatocytes still contain a low but significant number of mRNA molecules, only a few hepatocytes concentrated around the centrilobular veins stain for the protein. These AFP-positive hepatocytes very probably correspond to the few perivenous hepatocytes still displaying a strong hybridization signal for AFP mRNA on the adjacent sections. One likely explanation might be that the low level of AFP corresponding to the few transcripts detected in the periportal areas cannot be revealed under our immunoperoxidase working conditions (i.e., the use of light microscopy only).

The physiological significance of the present findings of a zonal heterogeneity in the level of AFP gene expression in the liver is not known. A link has been postulated to exist...
between proliferation and AFP-synthesizing capacity of hepatocytes (29). An explanation, therefore, might be the existence of a higher rate of cell proliferation in the perivenous area than in the other parts of the liver lobule, leading to a preferential expression of the AFP gene in the former zone. The results of in vivo [3H]thymidine injection experiments
in rats during the first two postnatal weeks obtained by Le Bouton (19) do not favor such a mechanism, since they showed a low proliferative activity of perivenous hepatocytes as compared to that of periportal and mediodlobular hepatocytes. On the other hand, Gleiberman et al. (13) have suggested that modulation of AFP synthesis during the early postnatal period depended on specific cell-to-cell interactions which vary during the progressive organization of the liver structure into definitive trabecular liver cell plates. Our observations would fit with this suggestion, as the appearance of the zonal heterogeneity is first recorded around the first week of postnatal life, at a time when the liver cell plates begin to assume the adult configuration (19); in contrast, no such heterogeneity is observed in the late fetus and the young neonates (1- and 5-d-old) when the liver is still constituted of irregularly arranged glandular-like structures and thick liver cell plates, respectively (19). Other factors might also be postulated. Glucocorticoid hormones have been proposed to exert an inhibitory effect on AFP gene transcription in vivo and in vitro (5, 15, 32). As it was suggested above for ALB, one might speculate that the quantitative differences of AFP gene transcription within the liver lobule are the result of an elective action of these hormones on the different lobular zones.

This paper is dedicated to the memory of José María Sala-Trepat prematurely deceased in October 1985 while this manuscript was in preparation. We thank Mrs. A. M. Durand-Schneider for her help in preparing the photographs. We are grateful to Drs. J. L. Danan and J. L. Nahon for critical reading of the manuscript. A special thanks is due to Dr. J. L. Salzmann for helpful suggestions.

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