Cell Lineages and Oval Cell Progenitors in Rat Liver Development

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ABSTRACT

We determined whether the formation of the hepatic primordium in the rat is associated with the expression of liver-specific markers. Further, we examined the origin of intra- and extrahepatic bile ducts and tried to establish whether there are cell types in the developing liver that might correspond to "stem-like" cells ("oval cells") that proliferate during carcinogenesis and toxic injury in adult livers. Using in situ hybridization and immunohistochemical methods, we show that α-fetoprotein (AFP) mRNA is detected in cells of the ventral foregut at 10.5 days of development and that the protein is first detected 1 day later. Thus, AFP transcription occurs before liver morphogenesis, and translation of the protein is first detected when liver cords are being formed, indicating that AFP expression in endodermal cells signals their commitment toward the liver lineage. Although albumin is considered a trait of differentiated hepatocytes, its mRNA was first detected just 1 day later than the AFP message. An analysis of the expression of lineage-specific cytokeratins (cytokeratins 7, 9, 18, and 19), surface markers, and histochemical determination of γ-glutamyl transferase activity and glycogen revealed that (a) hepatoblasts undergo gradual maturation throughout liver development, (b) AFP- and albumin-containing hepatoblasts gave rise to intra- and extrahepatic bile ducts, and (c) hepatoblasts forming primitive intrahepatic bile ducts during liver development have markers similar to those expressed by stem-like cells that proliferate during liver carcinogenesis.

INTRODUCTION

The morphological development of the liver from the ventral foregut endoderm has been described in detail in vertebrate embryos (1, 2). The liver primordium has both endodermal and mesodermal components, the first giving rise to hepatoblasts (3) to form the hepatic parenchyma while sinusoidal-lining cells and connective tissue components originate in the mesenchymal tissue invaded by the liver cords (3). Various aspects of liver development have received considerable emphasis including the inductive interactions between mesenchyme and epithelium (3, 4), the emergence of various molecular markers of liver cell maturation (5–9), the sequential appearance of enzymes involved in various hepatic functions (10), and the origin and development of intra- and extrahepatic biliary structures (11–13). The analysis of the expression of AFP during liver development in rodent and human embryos is of special interest because this protein is present only in very small amounts in the adult liver but is expressed in relatively large quantities during hepatocarcinogenesis and in most liver neoplasms (reviewed in Ref. 14). Moreover, it has been established in the past few years that in most models of experimental hepatocarcinogenesis, the cells that express AFP and its mRNA at the early stages of the process are not typical hepatocytes but are instead less mature cells that can function as progenitors for the hepatocytic and ductal lineages (15–18). These findings, together with the observation that cells of presumptive biliary origin but capable of AFP expression proliferate in liver injury produced by diverse agents (19–21), give particular importance to studies of the derivation and maturation of biliary structures during liver development (13, 22, 23).

Given this background, we conducted studies to examine cell lineages during rat liver development. The basic premise for this study is that the identification and characterization of precursors for mature hepatocytes and bile duct cells during embryonic development are essential for the understanding of both liver morphogenesis and the cellular aspects of toxic injury and neoplastic growth in the adult liver. We have focused on the following goals: (a) determining whether the formation of the liver primordium from endodermal cells is associated with the expression of tissue specific markers; (b) identifying maturation changes in hepatoblasts from 10 days of gestation to birth; (c) establishing the origin of intrahepatic bile ducts and searching for progenitor cells that express both hepatocyte and bile duct markers; and (d) identifying the origin and steps in the development of extrahepatic bile ducts. For these studies, we used in situ hybridization and immunohistochemical techniques to detect AFP and ALB mRNAs and the encoded proteins. We also analyzed by immunohistochemical methods the expression of cytokeratins and cell surface markers that are expressed in different cell types in adult rat liver and used histochemical methods to demonstrate GGT activity and glycogen. We found that AFP mRNA is made by endodermal cells before the start of liver morphogenesis and that ALB mRNA appears 1 day later when cord formation starts. We show that hepatoblasts at 15 to 18 days of development give rise to intrahepatic bile ducts and that cells in these structures continue to express AFP, albumin, and other hepatocyte markers as they begin to acquire phenotypic traits typical of ductal cells. These "dual phenotype" cells are similar to cells that proliferate in preneoplastic growth of adult rat liver.

MATERIALS AND METHODS

Animals. Embryos from Fischer 344/CrlBR rats (Charles River Breeding Laboratories) were used. Pregnancy was determined by the presence of sperm in the vaginal smear. The noon of the first day at which sperm was detected was considered as 0.5 days of gestation.

Demonstration of AFP and ALB by Immunohistochemistry. AFP and ALB localization in fetal liver was done as previously described (12, 24). Livers (after 15.5 days of gestation) or whole embryos (10.5 to 14.5 days of gestation) were fixed overnight in a mixture of cold 95% ethanol and glacial acetic acid (99:1, v/v) and embedded in paraffin. Incubation with the primary antibodies, sheep anti-rat AFP antiserum (Nordic Laboratories; 1/100 dilution) and rabbit anti-ALB antiserum (Organotechnic; 1/100 dilution), was done for 1 h at room temperature. The avidin-biotin complex method was used for AFP staining (anti-sheep immunoglobulin G biotinylated antibodies; Nordic; 1/100 dilution). For ALB staining the PAP procedure was used (goat anti-rabbit...
immunoglobulin G antibodies and rabbit PAP complex). Controls for unsp
specific staining were done using the same procedures but adding excess
purified AFP or ALB to the primary antibody solution.

Detection of AFP and ALB mRNAs by in situ Hybridization. Tissues
were frozen in n-hexane cooled in a dry ice-ethanol bath or fixed in
paraformaldehyde and embedded in paraffin. Hybridization with 35S-
labeled probes was as described by Evarts et al. (25) except that the
hybridization reaction was carried out at 43°C. Slides were coated with
NTB-2 emulsion, exposed for 1 to 14 days, developed, and stained with
hematoxylin and eosin.

The probes used for AFP mRNA detection were riboprobes (positive
probe and negative control) derived from plasmid RA1 (a gift of Dr. S.
Nishi) that contained a nearly full-length fetal rat AFP complementary
DNA clone. Two fragments were excised from this plasmid (700-base
pair PstI/BamHI and 800-base pair BamHI/PstI fragments) encoding the
5' end and the central portion of fetal rat AFP mRNA, respectively.

These fragments were ligated into the corresponding sites of the trans-
scription vector pBSM13+ (Stratagene Cloning Systems) in opposite
orientations in relation to the T7 polymerase promoter for the construc-
tion of antisense (700 base pairs) and sense (800 base pairs, negative
control) probes. The plasmids (pBASF700 and pBASF800) were linear-
ized and transcribed with T7 RNA polymerase as described by the
supplier (Stratagene), using 8-8uCi [35S]UTP (5 uCi final UTP concen-
tration). After digestion with DNase I, the 35S-labeled RNA probes
were subjected to limited alkaline hydrolysis (26) to obtain fragments
of 75 to 200 base pairs.

Riboprobes (sense and antisense) for ALB mRNA were derived from
the mouse ALB complementary DNA sequences of the pmalb2 plasmid
(27). The 700-base pair HindIII fragment was removed from pmalb2 and
subcloned in both orientations into the HindIII site of pGEM-3
(Promega Corp.). Plasmids pGmAlb2-1 (positive probe) and pGmAlb2-
4 (negative control) were linearized with BamHI. In vitro transcription
and alkaline hydrolysis were as described for the construction of AFP
riboprobes, except that Promega buffer and SP6 polymerase were used
in the transcription reaction. Hybridization reactions with antisense
and sense (control) probes were run in parallel in each experiment.
Further controls for hybridization with antisense probes were done by
treating the sections with 100 gg/ml of RNase A (Sigma) for 30 min
at 37°C before hybridization.

Immunohistochemical Methods. Whole embryos at 10.5 to 14.5 days
of gestation and livers from 15.5 days of gestation to the postnatal
period were frozen in n-hexane cooled in a dry ice-ethanol bath,
sectioned at 8 pm in a cryostat, and fixed in cold acetone for 10 min at
-20°C for staining of cytokeratins and cell surface markers. The fol-
lowing antibodies were used: (a) mouse monoclonal antibodies against
human cytokeratins 7, 8, 18, and 19 (designated CK7, CK8, CK18,
and CK19; Amersham Corp.; 1/10 dilution); (b) mouse mono-
clonal antibodies against rat CK5, 6, and 10, and 55,000 cytokeratins (a
gift from Dr. N. Marceau; 1/500 dilution) (28, 29); (c) guinea pig polyclonal
antibody against cow hoof prekeratin that recognizes a M, 52,000 rat
keratin (a gift from Dr. N. Marceau; 1/200 dilution); (d) rabbit
polyclonal antibody against calf muzzle keratin (Dako Corp.; 1/300
dilution); and (e) mouse monoclonal antibodies HES6 and BDS7 that
recognize surface antigens of, respectively, hepatocytes and ductal cells
in adult rat liver (a gift of Dr. N. Marceau; 1/200 dilution for HES6
and undiluted for BDS7). Embryos and livers were incubated with sec-
tod antibodies for 1 h at room temperature, and, after thorough washing
with 0.1 M PBS, the sections were incubated with secondary antibodies also for
1 h at room temperature. The secondary antibodies used were (a) for
mouse primary antibodies, FITC- or peroxidase-labeled goat antibodies
(Cappel and Dako Corp.; 1/50 dilution); (b) for guinea pig primary
antibodies, FITC-labeled rabbit antibodies (Dako; 1/50 dilution); and
(c) for calf muzzle keratin as the primary antibody, goat anti-rabbit
antibodies (Jackson ImmunoResearch; 1/50 dilution, 24 h incubation
followed by rabbit PAP complex at 1/100 dilution, incubated for 30
min at room temperature). After washing, slides were mounted in
parallel with control sections in which the primary antibody was re-
placed by PBS or normal rabbit serum (Jackson). Sections stained with
fluorescent dyes were examined in a Zeiss fluorescence microscope;
peroxidase-treated sections were stained with DAB. Some sections were
stained with both HES6 and calf muzzle keratin polyclonal antibody.

For this procedure, sections were incubated with a solution containing
the two antibodies, followed by incubation with a mixture containing
FITC-labeled anti-mouse immunoglobulins and goat anti-rabbit anti-
body. After examination of HES6 staining by immunofluorescence,
sections were incubated with rabbit PAP and stained by the DAB
reaction.

Lectin Binding. Sections were incubated with biotinylated PNA (50
µg/ml; Vector Laboratories) for 1 h at room temperature and then with
avidin-biotin complex (Vector) according to the manufacturer's instruc-
tions. Control sections were incubated with PNA solution containing
0.1 M lactose (Sigma Chemical Co.).

GGT Activity and Glycogen. GGT activity was demonstrated with
the method of Rutenberg et al. (31). Glycogen was visualized with the
periodic acid-Schiff reaction.

Transplantation of Fetal Liver Fragments. Fragments from livers at
Day 13.5 of gestation were transplanted into the testes of Fischer 344
rats (140 to 160 g) as described previously (32). The testes of these
animals were removed after 2 mo, and the areas containing the trans-
plants (identified with a dissecting microscope) were fixed in Bouin's
fluid and embedded in paraffin.

RESULTS

Developmental Commitment: AFP and ALB Expression. The liver
originates from the ventral foregut endoderm in a thickened
area that appears at the anterior intestinal portal region at
10.5 days of gestation (1, 2). We wanted to determine how
soon during the process of formation of the liver primordium
whether endodermal cells express genes that indicate develop-
mental commitment to liver morphogenesis. We analyzed the
expression of AFP and ALB by in situ hybridization for the
demonstration of mRNAs and by immunohistochemistry for
the localization of the protein. At 9.5 to 10 days of development
(0 to 4 somites) neither protein nor their mRNAs could be
detected in the endoderm, although AFP mRNA was abundant
in the yolk sac (data not shown). At 10.5 days of development,
when thickening of the endoderm in the ventral foregut region
starts, AFP mRNA was clearly detected in areas of the ventral
endoderm that were in contact with the septum transversum
mesenchyme (Fig. 1). By 11.5 days, both AFP mRNA and the
protein were present in hepatoblasts. These cells formed cords
that extended from the foregut endoderm into the septum
transversum (Fig. 1). Both the immunohistochemical staining
of AFP and AFP mRNA detection by in situ hybridization were
specific as determined with appropriate controls that included
removal of or competing out the primary antibody for the
immunohistochemical method and the use of the opposite
orientation AFP riboprobe and RNase treatment for the in situ
hybridization procedure.

ALB mRNA was detected approximately 1 day later than
was AFP mRNA, at 11.5 days of gestation, when hepatic cords
are formed (Fig. 2). Expression of ALB and its message was
clearly detectable by 12.5 days, at which time almost all hepa-
toblasts contained AFP and ALB mRNA and stained for the
proteins. Some ALB staining was detected in endodermal cells
of the region of the liver primordium at 10.5 days of develop-
ment, but we could not completely exclude the possibility that
the staining was nonspecific.

Stages of Maturation of Hepatoblasts. We studied the expres-
sion of various cytokeratins and surface markers by immuno-
histochemistry and determined GGT activity and glycogen by
histochemical methods in the liver of rats during fetal
development and the perinatal period. We chose markers that
 distinguish hepatocytes from bile duct cells in adult rat liver.
The distribution of cytokeratins in these cell types is particularly

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Fig. 1. AFP localization during rat liver morphogenesis. A, general features of 10.5-day rat embryo (H & E, × 100); neural tube (1), foregut (2), liver primordium (3), septum transversum mesenchyme (4), and visceral yolk sac (5). B, AFP immunostaining in 11.5-day liver (× 250); arrows indicate AFP-positive hepatic cords. C and D, AFP mRNA at 10.5 days detected by in situ hybridization (C, bright field; D, dark field; × 250; paraffin-embedded sections; 2-wk film exposure). Large arrow, liver primordium; small arrow, septum transversum mesenchyme. E and F, AFP mRNA at 11.5 days detected by in situ hybridization (E, bright field; F, dark field; × 250; 1-day exposure). Large arrows, hepatic cords; small arrow, extrahepatic bile duct.

helpful for this analysis as adult hepatocytes contain only CK8 and CK18, while duct cells have CK7 and CK19 in addition to CK8 and CK18 (33, 34). The other markers (22) selected for study are also specific for either hepatocytes (cell surface component HES6 and glycogen) or bile duct cells (cell surface component BDS7 and GGT activity).

We found that during fetal development hepatoblasts show a gradual change in the expression of some of these markers. At 11.5 and 12.5 days of development, CK8 was the only cytokeratin uniformly expressed by hepatoblasts (Table 1; Fig. 3). The mouse monoclonal antibody prepared against Mr 55,000 rat cytokeratin gave an identical staining pattern as that obtained with human CK8 antibodies. Neither HES6 staining nor glycogen was detected at this stage of development, but the vast majority of the cells stained for CK18 and had GGT activity (Table 1), although the expression of these two markers varied from cell to cell (Fig. 3). As development progressed, immature hepatocytes began to express HES6 at 15 to 16 days of development and glycogen at 19.5 days. Expression of CK18 and GGT activity remained patchy until birth, but during the postnatal period GGT activity was lost and CK18 staining became uniform (Table 1).
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Fig. 2. ALB mRNA localization during rat liver morphogenesis. Detection of ALB mRNA by in situ hybridization at 11.5 days (A and B, × 250; 2-day film exposure) and 13.5 days (C and D, × 125; 2-day exposure) of gestation. B and D are dark-field photographs to demonstrate silver grains in the same sections shown in A and C, respectively. Large arrow, liver parenchyma; small arrow, extrahepatic bile duct (determined by serial sections that showed the connection from liver to gut).

Table 1 Markers of hepatocyte development as detected by immunohistochemical staining or enzyme histochemistry

<table>
<thead>
<tr>
<th>Days of development</th>
<th>10–11.5</th>
<th>12–17</th>
<th>17.5–19</th>
<th>19.5–NB</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>AFP</td>
<td>AFP</td>
<td>AFP</td>
<td>ALB</td>
<td>ALB</td>
</tr>
<tr>
<td>CK8</td>
<td>ALB</td>
<td>ALB</td>
<td>CK8</td>
<td>CK8</td>
<td>CK8</td>
</tr>
<tr>
<td>CK18</td>
<td>CK18</td>
<td>CK18</td>
<td>CK18</td>
<td>HES6</td>
<td>Gly</td>
</tr>
<tr>
<td>GGT*</td>
<td>GGT</td>
<td>GGT</td>
<td>GGT</td>
<td>Gly</td>
<td>Gly</td>
</tr>
<tr>
<td>HES6</td>
<td>HES6</td>
<td>HES6</td>
<td>HES6</td>
<td>Gly</td>
<td>Gly</td>
</tr>
</tbody>
</table>

* NB, newborn; Gly, glycogen.

* CK8 staining was identical to that of a monoclonal antibody against rat M, 55,000 cytokeratin.

The markers listed stained 75 to 100% of cells.

and ALB mRNAs were detected by in situ hybridization in 25 to 50% and 10% of primitive bile duct cells, respectively (Figs. 5 and 6). As development progressed, maturing bile ducts gradually expressed CK7 and CK19 and the cell surface marker BDS7 (Table 2; Fig. 4).

Primitive bile ducts continued to be formed throughout fetal development and the first 1 to 2 wk after birth. Cells of mature bile ducts did not contain AFP and ALB mRNAs and did not stain for these proteins. Double staining in the same section with the hepatocyte surface marker HES6 and the bile duct marker poly-CK, or staining with HES6 and CK19 antibodies in consecutive sections of 17.5-day and neonatal livers, showed that both the hepatocyte and the ductal cell markers were present in the same cells in primitive ducts (Fig. 6). Two wk after birth, intrahepatic bile duct cells no longer stained with HES6 (Table 2). These results show that bile ducts originate from intermediate cells expressing hepatocyte and ductal markers present in the liver parenchyma near portal spaces starting at 15.5 days of development. Cells of primitive bile ducts undergo maturational changes during development but continue to express hepatocyte traits for 1 to 2 wk after birth.

The Origin of Intrahepatic Bile Ducts. Intrahepatic bile ducts develop from hepatoblasts surrounding the large vascular spaces (1, 2, 13, 23, 32, 35, 36). We wanted to determine whether intermediate cells containing markers for both the hepatocyte and ductal lineages would be present during liver development and be the progenitors of intrahepatic bile ducts. The major interest in searching for these cells is that they might correspond to the immature, progenitor-type cells that proliferate during liver carcinogenesis in adult rats. At 15.5 days of development, hepatoblasts around the large portal vein close to the hilus began to form pearl-like structures, as described by Van Eyken et al. (13). These primitive bile ducts were heavily stained by both CK18 and CK8 antibodies and had high GGT activity (Table 2; Fig. 4). CK18 was a particularly good marker for these cells because of the strong staining of primitive biliary structures in comparison with parenchymal cells. In addition, some cells in these structures expressed CK19 (a bile duct marker), and 50 to 75% of cells contained AFP and ALB. AFP and ALB mRNAs were detected by in situ hybridization in 25 to 50% and 10% of primitive bile duct cells, respectively (Figs. 5 and 6). As development progressed, maturing bile ducts gradually expressed CK7 and CK19 and the cell surface marker BDS7 (Table 2; Fig. 4).

Primitive bile ducts continued to be formed throughout fetal development and the first 1 to 2 wk after birth. Cells of mature bile ducts did not contain AFP and ALB mRNAs and did not stain for these proteins. Double staining in the same section with the hepatocyte surface marker HES6 and the bile duct marker poly-CK, or staining with HES6 and CK19 antibodies in consecutive sections of 17.5-day and neonatal livers, showed that both the hepatocyte and the ductal cell markers were present in the same cells in primitive ducts (Fig. 6). Two wk after birth, intrahepatic bile duct cells no longer stained with HES6 (Table 2). These results show that bile ducts originate from intermediate cells expressing hepatocyte and ductal markers present in the liver parenchyma near portal spaces starting at 15.5 days of development. Cells of primitive bile ducts undergo maturational changes during development but continue to express hepatocyte traits for 1 to 2 wk after birth.

The Origin of Extrahepatic Bile Ducts. It has been reported that extrahepatic bile ducts develop independently from intrahepatic duct formation (13, 37, 38). We have confirmed these observations and further analyzed the formation of extrahepatic...
Fig. 3. Stages in hepatocyte maturation. A, CK8 staining of cells in hepatic cords (arrow) at 11.5 days of development. B, GGT activity in parenchymal (large arrow) and perivascular (small arrows) cells at 17.5 days. C and D, CK18 staining in 19.5-day and neonatal liver, respectively, demonstrating heavily positive duct structures around a portal vein; parenchymal cells in the background are lightly stained. Thick arrow, intrahepatic bile ducts; thin arrow, portal vein. E and F, HES6 staining in 14.5- and 19.5-day liver, respectively. Note absence of staining in E (14.5 days) and staining at 19.5 days in both parenchymal and perivascular cells. Arrow, portal vein. All panels, × 250; A and E, immunoperoxidase method, counterstained with hematoxylin; C to F, immunofluorescence.

Table 2 Markers of intrahepatic bile duct development as detected by immunohistochemical staining or enzyme histochemistry

<table>
<thead>
<tr>
<th>Days of development</th>
<th>Newborn*</th>
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<tr>
<td>15.5*</td>
<td>Immature ducts</td>
</tr>
<tr>
<td>AFP (50-75%)</td>
<td>AFP (10-25%)</td>
</tr>
<tr>
<td>ALB (50-75%)</td>
<td>ALB (&lt;5%)</td>
</tr>
<tr>
<td>CK8</td>
<td>CK8</td>
</tr>
<tr>
<td>CK18</td>
<td>CK18</td>
</tr>
<tr>
<td>Poly-CK</td>
<td>CK7 (10%)</td>
</tr>
<tr>
<td>GGT</td>
<td>CK19 (25-50%)</td>
</tr>
<tr>
<td>Poly-CK</td>
<td>Poly-CK</td>
</tr>
<tr>
<td>GGT</td>
<td>GGT</td>
</tr>
<tr>
<td>HES6 (10-25%)</td>
<td>HES6 (&lt;5%)</td>
</tr>
</tbody>
</table>

* Newborn liver contains both immature and mature ducts. Mature ducts are the only structures found in adult livers.

Intrahepatic bile ducts develop from hepatoblasts located near the vascular spaces at 15.5 days of development. The markers listed are for hepatoblasts that form primitive bile ducts.

* CK8 staining was identical to that observed using monoclonal antibodies against rat M, 55,000 cytokeratin.

Ducts. These ducts were first detected at about Day 11 of development; the cells forming the common bile duct contained AFP and AFP mRNA (Fig. 8). The hepatic duct originated at 15 to 16 days of development from hepatoblasts that contained AFP mRNA, AFP, and ALB. At 19.5 days of development, cells of extrahepatic ducts no longer expressed AFP or ALB. Starting at 11.5 days of development, extrahepatic bile ducts stained for CK8, poly-CK, and CK19 and, at later stages (19.5 days, and newborn animals), they stained for CK7, CK8, CK18, CK19, and poly-CK, as well as the lectin PNA (Table 3; Fig. 8). The results indicate that portions of the extrahepatic bile ducts originate from AFP-producing hepatoblasts, while other structures (hepatic duct) emerge a few days later from AFP- and ALB-containing cells.

DISCUSSION

An important issue in the analysis of development is the mechanism by which cells from the various layers become determined or committed to form a specific tissue. In particular,
Fig. 4. Development of intrahepatic bile ducts. Demonstration of cytokeratins in periportal cells forming primitive bile ducts (arrows). Staining of 17.5-day liver for CK8 (A) and CK19 (B). Staining of neonatal liver for CK8 (C, hepatocytes lightly stained), CK18 (D), CK7 (E, hepatocytes counterstained with hematoxylin are CK7 negative), and CK19 (F). All panels, × 250.

it is important to find out the time at which commitment takes place and whether or not the expression of tissue-specific markers precedes morphogenesis of that tissue. Specifically, we wanted to determine whether the start of transcription and translation of the AFP message would occur in endodermal cells of the ventral foregut before the liver was formed. Furthermore, we wanted to find out when ALB expression was first detected during liver development.

During rodent development, AFP is expressed in the visceral cells of the yolk sac by 6 to 8 days of gestation and several days later in the fetal liver (5, 39). Most studies have involved measurements of liver AFP mRNA by nucleic acid hybridization, starting at 14 to 15 days of development in rats or mice (6–8). Muglia and Locker (6) could not detect AFP message by dot-blot hybridization with RNA preparations obtained from the foregut region of 10-day-old rat embryos, while at 12 days, both AFP and ALB mRNAs were apparently present. The single in situ hybridization study of AFP and ALB expression during rat liver development, an analysis of the expression of both genes in late gestation and the perinatal period, showed that all hepatocytes of 17- to 19-day-old rat fetuses contain AFP and ALB messages (40). We show that the AFP message is detectable by in situ hybridization in cells of the ventral foregut in the anterior intestinal portal region at 10.5 days of development (9 to 14 somite stage) and that the protein is found 1 day later. This indicates that AFP transcription occurs before liver morphogenesis and that translation of the mRNA into the protein is first detected when liver cords are being formed and extended into the mesenchyme. In contrast, ALB mRNA was first demonstrated at 11.5 days of development, coincident with liver cord formation, while the protein was detected 1 day later, when cord formation is completed. Endoderm of the foregut region becomes determined for liver formation as early as the 5-somite stage in chick embryos, and the determination requires inductive interaction between the ventral foregut endoderm and the hepatocardiac mesenchyme (4). Based on the time of appearance and localization of the AFP message revealed by our experiments, AFP transcription seems to be one of the earliest markers of liver-specific gene expression in determined endodermal cells. It is likely that the start of AFP and ALB gene transcription during liver morphogenesis requires tissue-specific transcription activators (reviewed in Ref. 41). If this is the
Fig. 5. Localization of AFP and albumin mRNAs in hepatoblasts forming primitive bile ducts. AFP (A and B; arrows indicate the alignment of the sections) and ALB mRNAs (E and F) detected by in situ hybridization in periporal cells forming duct structures at 16.5 days of development. C and D (controls), hybridization with AFP probe in 16.5-day liver sections pretreated with RNase. A, C, and E, bright field; B, D, and F, dark field photographs. All panels, x 500; 2- and 3-day film exposure for AFP and ALB message, respectively.

Fig. 6. Origin of intrahepatic bile ducts from ALB- and HES6-positive cells at 17.5 days of development. A and B, biliary ducts formed by ALB mRNA-positive hepatocytes (arrows); biliary cells at the opposite side of the ducts are ALB mRNA negative (arrowhead); (A, bright field; B, dark field; x 500; 3-day film exposure). C and D, double staining with polyclonal anti-CK (C) and HES6 (D) in the same section (x 250). Large arrows indicate peripoortal cells forming ducts around the portal vein (small arrows).
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Fig. 7. Fragment from 13.5-day liver transplanted into the testes of a syngeneic rat. The testes were examined 2 mo after transplantation and show large hepatocytes (arrowheads) and differentiated intrahepatic bile ducts (arrow). H & E, × 250.

In this case, the necessary factors must be present before or at the time when AFP mRNA is first detected in foregut endodermal cells at the 9- to 14-somite stage of rat and mouse development.

Previous work (12) demonstrated by immunohistochemical methods in the developing mouse that AFP is present in some endodermal cells of the anterior intestinal portal region, particularly in the cranial diverticulum. It was also observed that in mice, ALB was detectable at 10.5 to 11.5 days of gestation, approximately 1 day later than AFP. Although the previous studies did not include the analysis of AFP and ALB transcripts by in situ hybridization, our work in rats entirely agrees with the published results.

AFP-producing hepatoblasts acquired hepatocyte-specific characteristics in a sequential pattern that included production of ALB, expression of HES6, and finally glycogen accumulation. Albumin, which is considered a marker for differentiated hepatocytes, is already produced by hepatoblasts at 12 days of development; in contrast, HES6 staining appeared several days later, at 15.5 days of development. The changes in the pattern of cytokeratin expression in hepatoblasts were relatively minor throughout development, with the exception of cells located near the vascular spaces (see below). Other investigators have shown that both in vivo and in culture, enzymes associated with various liver functions also appear in a stepwise fashion during liver development (42, 43).

Van Eyken et al. (13) and Shiojiri (12, 24, 32) demonstrated that intrahepatic bile ducts develop from immature cells located

Fig. 8. Development of extrahepatic bile ducts from AFP-containing cells. Localization of AFP mRNA in cells forming extrahepatic bile ducts at 16.5 days of development (A, bright field; B, dark field; 2-day film exposure). C and D are controls for AFP hybridization using a section from 16.5-day liver pretreated with RNase. E and F, staining of extrahepatic bile ducts with CK7 (17.5 days) and PNA (neonatal liver), respectively. All panels, × 250.
Differential of Rat Liver Cells

Table 3 Markers of extrahepatic bile duct development as detected by immunohistochemical staining or enzyme histochemistry

<table>
<thead>
<tr>
<th>Days of development</th>
<th>11.5*</th>
<th>15-16</th>
<th>19.5/newborn</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP (50-75%)</td>
<td>AFP (50-75%)</td>
<td>CK7</td>
<td></td>
</tr>
<tr>
<td>CK8 (25-50%)</td>
<td>CK8</td>
<td>CK18</td>
<td></td>
</tr>
<tr>
<td>Poly-CK</td>
<td>CK18</td>
<td>CK19</td>
<td></td>
</tr>
<tr>
<td>PNA</td>
<td>Poly-CK</td>
<td>PNA</td>
<td></td>
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<tr>
<td>BDS7</td>
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* Common bile duct.
† Hepatic duct.
‡ AFP mRNA was present in 50 to 75% of cells at this stage.

near the large vascular spaces. We confirmed and extended these observations with the use of a large number of markers and in situ hybridization techniques. One of our goals was to search for cells that would have a pattern of expression of AFP, ALB, cytokeratin, and cell surface markers similar to that of epithelial cell populations that proliferate during liver injury and carcinogenesis. Such cells have markers of both hepatocyte and ductal lineages (30, 44, 45) and apparently constitute a reserve stem cell compartment in adult rat liver (46-49). We found that cells of immature bile ducts expressed hepatocyte markers (AFP, ALB, HES6) during development and for 1 to 2 wk after birth as they gradually acquired bile duct markers (CK7, CK19, poly-CK, and BDS7). Based on the simultaneous expression of hepatocyte and duct markers (AFP, ALB, CK7, CK19), we suggest that "oval cells" that proliferate at the early stages of hepatocarcinogenesis as well as AFP-containing "ductal cells" detected after galactosamine injury correspond to cells of primitive bile ducts that appear at Day 15.5 to Day 17.5 of development and express hepatocyte and bile duct markers.

The types of markers expressed by primitive bile duct cells change during development. For instance, at 17.5 days of gestation, AFP predominates over CK7 but, at birth, CK7 expression is much higher. Similarly, single cells in oval cell populations of preneoplastic or injured liver express hepatocyte and duct markers in variable proportions, presumably indicating different stages of maturation and lineage commitment (16, 30).

Germain et al. (22) showed that, in culture, liver cells from 12-day fetal rats express markers corresponding to the hepatocyte or ductal lineages, depending on growth conditions. Here we show that fragments of 13.5-day fetal liver transplanted to the testes of young adult rats give rise to large, mature hepatocytes as well as bile duct structures. The transplanted fragments were obtained several days before the emergence of intrahepatic bile ducts and contained only immature parenchymal cells. In vivo, only those hepatoblasts surrounding the vascular spaces develop into duct cells and express markers for both lineages. However, some hepatocytes scattered through the parenchyma distant from the vascular spaces also contain some of these markers. Although both the perivascular and parenchymal hepatoblasts might be dual lineage progenitors, duct formation in liver development may require the inductive interaction between hepatoblasts and the matrix components of the venous spaces (32). These results are in general agreement with the studies of Shah and Gerber (50, 51), who found that laminin might be required for bile duct differentiation in humans. They also suggested that some immature duct structures persisting ...

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Cell Lineages and Oval Cell Progenitors in Rat Liver Development

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