

Aquisition of Antigens Characteristic of Adult Pericentral Hepatocytes by Differentiating Fetal Hepatoblasts In Vitro

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Abstract. Antigens specific to pericentral hepatocytes have been studied in adult mouse liver, during fetal development, and in cultured fetal hepatoblasts. Antibody reactive with glutamine synthetase stained all fetal liver cells but almost all cells lost this antigen after birth; only a single layer of pericentral cells retained it in adulthood. In contrast, monoclonal antibodies to major urinary protein (MUP) did not detect the antigen until ~3 wk after birth, after which time the cells within 6–10 cell diameters of the central veins were positive. Cultured fetal liver cells from embryos at 13 ± 1 d of gestation were capable of differentiating in vitro to mimic events that would occur had the cells remained in the animal. About 10–20% of the ex-

planted cells grew into clusters of hepatocyte-like cells, all of which stained with albumin antibodies. MUP monoclonals were reactive with one-half of the differentiated fetal hepatocytes. Glutamine synthetase was present in all hepatocytes after several days in culture and gradually decreased and remained in only occasional cells, all of which also contained the MUP antigen.

These findings suggest that a sequence of gene controls characterizes expression of specific genes in developing liver, and that differentiating fetal hepatoblasts are capable of undergoing similar patterns of gene activity in culture.

THE adult liver is arranged in acini with the afferent blood supply from the portal vein and the portal artery entering branching vessels at the microscopic level deliver blood to capillary-sized vessels, termed sinusoids, which conduct blood between cords (or plates) of hepatocytes to empty into a central vein. Thus hepatocytes are divided into two zones or regions: periportal (close to the portal afferent circulation) or pericentral (close to the efferent central vein) (51). It is widely accepted that all hepatocytes in rodents and humans contain certain proteins (for example albumin), while other proteins are found at higher levels in cells of one or the other zone (4, 8, 23, 24, 32, 33, 42, 55, 58, 59), perhaps related to the higher nutrient, hormone, and/or oxygen content of blood in periportal compared with the pericentral region. However recent work using immunohistochemical labeling shows a distinct pericentral location for a number of different detoxification enzymes whose regulation is not obviously related to nutrient or oxygen supply (2, 52, 62). These antigens are present either exclusively or predominantly in the 6–10 cell layers immediately surrounding the central vein in adult rodents. In addition, antibodies to glutamine synthetase show this enzyme to be present in rats in a single layer of pericentral cells (22). To supplement our studies on gene expression in mouse hepatocytes, which have emphasized the importance of cell surface contacts in maintaining hepatocyte specific transcription (12), and to begin to examine hepatocytes during fetal

development, we prepared a series of mAbs against mouse liver tissue. Many of the first group of mAbs we obtained recognized antigens that were present only in pericentral cells. Using these antibodies we selected cDNA clones from an expression library of mouse liver cDNA constructed in our laboratory. At least eight of the pericentral antigens are related to the major urinary proteins (MUP)¹ of mice. This group of mouse proteins is related to the rat α 2u-globulin, which is known to be found in the pericentral region (2). We have compared the localization of generally distributed antigens (e.g., albumin) with the adult pericentral antigens and with glutamine synthetase during fetal and neonatal development in the mouse. Striking differences in the regional distribution within the liver acinus of various antigens, particularly glutamine synthetase, were observed during ontogeny. All late fetal hepatocytes reacted with albumin and glutamine synthetase but not with any of 12 mAbs that detect pericentral antigens in the adult. Glutamine synthetase became limited in distribution within a few days after birth and the pericentral antigens were not detected until 2–3 wk after birth. This corresponds to the known time of appearance of the MUP proteins in mice, an event controlled at the level of transcription of the mRNAs (15).

To further examine what appears likely to be both positive and negative regulatory events in liver gene expression, cul-

1. Abbreviation used in this paper: MUP, major urinary protein.

tures of differentiating fetal hepatoblasts were studied using antibodies to glutamine synthetase and the pericentral antigens. In contrast to adult hepatocytes cultured under identical conditions, where liver-specific function becomes quickly compromised, differentiating fetal hepatoblasts underwent a program of protein appearance similar to normal development. The explants of 13-d hepatic buds first developed and then lost glutamine synthetase; the fall in glutamine synthetase was accompanied by a rise in pericentral antigens that persisted for several weeks.

Materials and Methods

Monoclonal Antibody Preparation

Monoclonal antibodies specific for antigens present in adult mouse liver were prepared by standard methods (35, 41). Male Sprague-Dawley rats were immunized intraperitoneally at monthly intervals with a crude mouse liver membrane fraction (29) in Freund's adjuvants. After the third immunization, the spleenocytes were isolated and polyethylene glycol fused with SP2 myelomas and cultured in high glucose (4.5 g/l) DME supplemented with 10% FBS, 10% NCTC 109 medium, 2 mM glutamine, 1 mM sodium pyruvate, 200 U/l insulin, 100 U each penicillin and streptomycin per ml, and 50.0 mg/l garamycin in 96-well plates under hypoxanthine-aminopterin-thymidine selection. The resulting hybridomas were screened by dot-immunobinding assay (27), and those giving a positive result on a dot of crude liver membranes but not on a dot of crude common tissue membranes (a combination of heart, lung, kidney, and brain) were chosen for subcloning and further study.

Polyclonal Antisera

Sheep anti-mouse albumin was purchased from Cappel Laboratories, Inc. (Cochranville, PA). Rabbit anti-rat glutamine synthetase was prepared by injection of purified rat glutamine synthetase (45, 56).

Primary Fetal and Adult Liver Cultures

Fetal liver cultures from staged fetuses at $\sim 13 \pm 1$ d of gestation or at term (~ 19 d gestation) were prepared by a modification of the method of Leffert and Paul (38, 39). Dams were anesthetized with ether and the uterine horns removed. Under sterile conditions, the fetuses were removed from the uterus and immediately decapitated. Fetal livers were then dissected (for 13-d fetuses, the midsection of the fetus containing the liver bud as well as some other surrounding tissues was taken) and digested with frequent, brief, high-speed vortexing for 10 min (13 d) or 20–25 min (term) at 37°C in 1.5 mg/ml type II collagenase in arginine-free, ornithine supplemented (67.24 mg/l) high glucose (4.5 g/l) DME. Undigested debris was allowed to settle to the bottom of a conical tube, the cell suspension was drawn off, an equal volume of serum-containing medium was added, and the cells collected by centrifugation (5 min at 200 g), resuspended, and plated (5×10^6 in 2 ml of medium) in 35-mm plastic culture dishes containing sterile 22 \times 22 mm glass coverslips. The culture medium was supplemented with 10% dialyzed FBS, 10.0 mg/l cortisone, 200 U/l insulin, 50.0 mg/l garamycin, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were kept in a 37°C incubator with 5% CO₂ and allowed to attach for 6–12 h, then the nonadherent cell population was removed, and the dishes were rinsed once with medium, and refed. Thereafter the media was changed approximately once a week or as needed.

Primary cultures of adult mouse hepatocytes were prepared by collagenase perfusion as previously described (11). Cells were plated at 2.5×10^5 in 2 ml of medium under conditions equivalent to the fetal liver cultures.

Immunocytochemistry of Tissue Sections and Cultured Cells

Frozen sections of various tissues were prepared and processed by standard methods (5) for determination of antigen content. Adult mice were anesthetized with Nembutal and given a small amount of heparin intraperitoneally. A cannula was placed through the right atrium into the inferior vena cava with perfusion with PBS, pH 7.4, was followed by perfusion of freshly pre-

pared 4% paraformaldehyde in PBS. Liver and other organs were dissected out and postfixed in 4% paraformaldehyde for a few hours to overnight, then equilibrated in 30% sucrose in water at 4°C. Fetal tissues and whole fetuses were obtained in a manner similar to that used for cultures, and fixed for 1–2 d at 4°C in 4% paraformaldehyde, then equilibrated in sucrose solution. Tissues were embedded and frozen in OCT compound, and 7- μ m sections were cut and mounted on gelatinized slides. Nail polish wells were placed around each section and sections were incubated with 0.1–0.3 ml of blocking solution (5% normal goat serum in PBS) for 30 min, and then left overnight at room temperature or 4°C in 0.2–0.3 ml of primary antibody. Typically, primary antibody consisted of a 1:500 dilution of an antiserum in blocking solution with 0.1% Triton, or undiluted mAb tissue culture supernatants containing 0.1% Triton. The slides were washed by dipping in five changes of PBS, blocked again for 30 min, and 0.05 ml of a 1:50 dilution of secondary antibody was added (secondary antibodies were either rhodamine or fluorescein conjugates of F(ab)'; fragment goat anti-rat or anti-rabbit IgG heavy and light chain). The secondary antibody was left on for 2 h, the slides were again extensively washed in PBS, and then the coverslips were mounted using 50% glycerol/PBS. Sections were viewed and photographed using epi-fluorescent optics on a Nikon Diaphot inverted phase microscope.

Cultured cells were processed for immunofluorescence in a similar manner to the sections (5). Plates of cells were rinsed with PBS and fixed 30 min on ice in 4% paraformaldehyde. At room temperature, the fixative was removed and the cells were permeabilized with 0.1% Triton/PBS for 10 min, washed with PBS, and left in blocking solution 30 min. Primary antibody (2 ml, without detergent added) was left on overnight, and all subsequent steps were as for tissue sections.

Double labeling of both sections and cultured cells was achieved by simultaneous incubation with both a rat mAb and a rabbit polyclonal to albumin or glutamine synthetase, with subsequent simultaneous incubation of two secondary antibodies conjugated to different chromophores (fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-rat IgG).

Preparation of Double-stranded cDNA

Mouse liver total cellular RNA was prepared essentially as described by Chirgwin et al. (9). Total poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography as described by Maniatis et al. (43). 20 μ g mouse liver poly(A)⁺ RNA was denatured with 5 mM CH₃HgOH. First strand synthesis conditions were 0.2 mg/ml RNA, 25 mM Tris HCl pH 8.3, 50 mM KCl, 8 mM MgCl₂, 1.5 mM each nucleotide triphosphate, 30 mM 2-mercaptoethanol, 80.0 μ Ci/ml [³H(N)]dATP, 30 μ g/ml oligo dT (12–18), 100 μ g/ml Actinomycin D, 1.0 U/ml placental RNase inhibitor, and 6 U avian myeloblastosis virus reverse transcriptase per μ g RNA at 42°C for 1 h. Second strand synthesis was carried out using the *Escherichia coli* RNaseH/DNA ligase method as described (25). The average size of the cDNA was estimated to be 1,200 bp.

Addition of Eco RI Linkers, Ligation into λ gt11 Arms, and Packaging into λ Phage

The cDNA was methylated at internal Eco RI restriction sites with Eco RI methylase according to manufacturers specifications (New England Biolabs, Beverly, MA). 0.5 μ g of phosphorylated Eco RI linkers were ligated to 5 μ g cDNA as described by Maniatis et al. (43). The ligase was inactivated at 68°C, and linker oligomers cleaved from the cDNA by digestion with Eco RI. Eco RI linker monomers were removed by passing over a Sepharose CL-4B column (43). cDNA was ligated to phosphatase-treated λ gt11 arms (Vector Cloning Systems, San Diego, CA) at a molar ratio of 1:1.5 and a DNA concentration of 325 μ g/ml. Recombinant DNA was packaged using 1×10^7 phage/ μ g packaging extracts (Vector Cloning Systems). Greater than 95% of the recombinant phage contained cDNA inserts and recombinants were obtained at an efficiency of 4,000/ng cDNA.

Antibody Screening of Recombinant Phage

400,000 recombinant phage were absorbed onto *E. coli* strain Y1090, plated at a density of 25,000 plaques per 15-cm NZ YCM-ampicillin plate (43). Fusion proteins were induced and transferred to nitrocellulose as described by Young and Davis (64, 65). Filters were removed and washed three times in PBS for 15 min at 25°C, then blocked in PBS containing 10% goat serum (GS-PBS) for 30 min. Filters were incubated with a 1:500 dilution of pericentral mAbs in GS-PBS overnight (mAb tissue culture supernatants were concentrated by precipitation with 50% ammonium sulfate). Unbound

antibody was removed by washing the filters three times in PBS for 15 min, and the filters blocked for a second time in GS-PBS for 30 min. Filters were then incubated with a 1:1,000 dilution in GS-PBS of horseradish peroxidase-conjugated goat anti-rat IgG F(ab')₂ fragment for 2 h. The filters were washed three times in PBS for 15 min, then developed for 30 min in PBS with 0.01% H₂O₂ and a 1:5 dilution of 0.3% (wt/vol) 4-chloro-1-naphthol in methanol. Positive signals from the first antibody screen were plaque purified through four additional rounds of screening.

Isolation of Phage DNA and Sequencing

Purified recombinant plaques were amplified as plate stocks and used in large scale preparations of phage (43). Isolated phage DNA was digested with Eco RI and inserts cloned into pGEM-1. Additional subclones were constructed and sequenced using synthetic oligomers corresponding to the SP6 and T7 promoters on this vector, and the chain termination sequencing method of Sanger et al. (54).

Results

MUP Is a Pericentral Antigen

After immunization of rats with crude mouse liver plasma membrane preparations, hybridoma cells were prepared by a fusion of rat spleen cells with mouse myeloma cells (35, 41). 14 clonal cell lines were selected that produced antibodies reactive in a dot immunobinding assay (27) with extracts of adult mouse liver but not with extracts of brain, kidney,

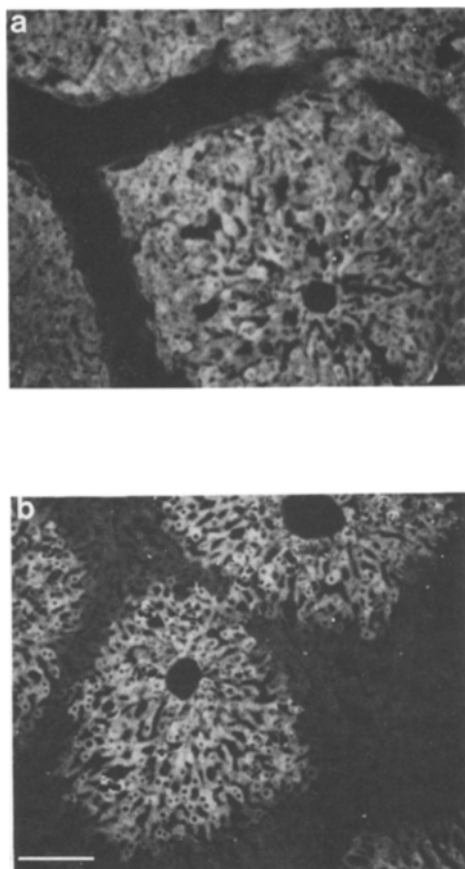


Figure 1. Adult mouse liver sections were labeled with polyclonal antisera to mouse albumin (a) or monoclonal antibodies from rat-mouse hybridomas selected against mouse liver membrane preparations (b), which show a distinctive pericentral localization. Bar, 184 μ m.

or spleen. Immunohistochemical staining of liver sections with the antibodies revealed that two of the 14 mAbs reacted with all of the hepatocytes in sections of male or female mouse liver. Antibodies against purified albumin (Fig. 1 a) or alpha-1-antitrypsin (data not shown), two prominent secretory products specifically synthesized by hepatocytes (42, 55), have a similar staining pattern. The remaining 12 mAbs only reacted with cells in the pericentral region of the liver as contrasted to the periportal region (Fig. 1 b). The staining extended ~6–10 cells away from the central vein and the most central and most peripheral of the stained cells were stained equally, that is, the boundary between stained and unstained cells was not gradual but sharply demarcated. Overall about half of the hepatocytes stained with these antibodies. Of this group of 12 antibodies, only one reacted with rat liver and none reacted with other mouse or rat tissues. Only one antibody of this group was capable of precipitating a specific protein; the size of the precipitated antigen was 18 kD, determined by gel electrophoresis (1, 57; data not shown).

As noted in the introduction, cytochrome P450, NADPH reductase, glutathione-s-transferases, and epoxide hydroxylase, all enzymes concerned with detoxification, are by immunologic tests found to have an exclusive or predominant localization in the pericentral area of the rat liver acinus (52, 62). However, none of these proteins has a molecular mass of 18 kD. To identify the protein(s) that were reactive with our mAbs we prepared a library of λ GT11 bacteriophages carrying cDNA inserts from copies of mouse liver mRNA. From this library several clones that produce proteins reactive with eight of the pericentral mAbs were chosen and the cDNA sequenced. The sequence proved to be that of the mRNA for the major urinary protein of mice (Fig. 2). There was one change at amino acid 235 (Thr to Asn) not previously reported in the literature.

Probably at least two different MUP antigens are represented in the group of antibodies reactive with pericentral cells based on results with regenerating liver. Sections of liver were prepared at 12, 24, 36, and 48 h and 7 d after partial hepatectomy and reacted with the panel of pericentral mAbs. Six of the antibodies reacted with pericentral cells during regeneration, while the other six mAbs that reacted with normal adult pericentral cells did not react with regenerating tissue (data not shown). In addition, examples of both types of mAbs recognize MUP cDNA fusion proteins produced in recombinant λ plaques. This suggests that at least two different epitopes in the pericentral region were recognized by the present collection of monoclonal antibodies. Using a cDNA clone to a type I MUP mRNA we earlier had found that both the transcription and steady-state levels of MUP sequences declined dramatically during postoperative liver regeneration (18). Therefore, since some antigens disappear postoperatively and others do not, it is likely that the mAbs recognize at least two different proteins or two different epitopes on two categories of MUP proteins.

Regional Localization of Glutamine Synthetase

A different and highly regional distribution for another liver protein is also known. Antibodies to pig retinal glutamine synthetase are known to react in adult rat liver with a single layer of cells around the central vein (22). Rat liver gluta-

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-25 ATATAAGGAC AAGCAAAGGG GCTGGGGAGT GGAGTGTAGC CACGATCACA AGAAAGATGT
      +1 mRNA start      8-1 start
36 GGTCTGTACA GACAGACAAT CCTATTCCTC ACCAAAATGA AGATGCTGCT GCTGCTGTGT
      Met start 13-1 start
96 TTGGGACTGA CCCTAGTCTG TGTCCATGCA GAAGAAGCTA GTTCTACGGG AAGGAACCTT

156 AATGTAGAAA AGATTAATGG GGAATGGCAT ACTATTATCC TGGCCTTTGA CAAAAGAGAA

216 AAGATAGAAG ATAATGGCAC CTTAGACTT TTTCTGGAGC AAATCCATGT CTTGGAGAAT
      Thr-Aaa
276 TCCTTAGTTC TTAATTTCCA TACTGTAAGA GATGAAGAGT GCTCCGAATT ATCTATGGTT
      Ser-Ser
336 GCTGACAAAA CAGAAAAGGC TGGTGAATAT TCTGTGACGT ATGATGGATT CAATACATTT

396 ACTATACCTA AGACAGACTA TGATAACTTT CTTATGGCTC ATCTCATTAA CAAAATGAT

456 GGGGAAACCT TCCAGCTGAT GGGGCTCTAT GGCCGAGAAC CAGATTGAGT TTCAGACATC

516 AAGGAAAGGT TTGCACAAC TATGTAGGAG CATGGAATCC TTAGAGAAAA TATCATTTGAC
      Glu-Lys
576 CTATCCAATG CCAATCGCTG CCTCCAGGCC CGAGAATGAA GAATGGCCTG AGCCTCCAGT
      stop
636 GTTAGTGGGA GACTTCTCAC CAGGACTCCA CCATCATCCC TTCCTATCCA TAGAGCATCC

696 CCAGTATAAA TTCTGTGATC TGCATTCCAT CCGTGTCTAC TGAGAAGTCC AATTCCAGTC

756 TATCCACATG TTACCTAGGA TACCTCATCA AGAATCAAAG ACTTCTTTAA ATTTCTCTTT
      T
816 GATATACCCA TGACAATTTT TCATGAATTT CTTCCCTTTC CTGTTCAATA AATGATTACC

876 CTTCACCTTA (A)n

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Figure 2. Sequence of cDNA clones that encode pericentral antigens of mouse liver corresponds to MUP. From a λ gt11 expression library single plaques were identified that produced antigen reactive with a mAb of the type used in Fig. 1 *b* to stain pericentral hepatocytes. The consensus sequence of MUP exons (10) is presented on the top line and pericentral cDNA clones 8-1 and 13-1 are on the bottom. The beginning positions of clones 8-1 and 13-1 are indicated by arrows (>). Nucleotide changes from the consensus are indicated with corresponding amino acid changes.

mine synthetase has been purified (56) and a polyclonal antibody to the protein (45) was reacted with mouse liver sections. As in the rat, a single layer of pericentral cells (Fig. 3 *c*) reacted, in contrast to the reaction of our mAbs with a layer of cells 6-10 deep around each central vein (Fig. 3 *b*).

Using different fluorescent chromophores (fluorescein and rhodamine) and several antibodies alone or in combination, at least three patterns of different antigen distribution and presumably gene expression were detectable in adult mouse liver: (a) all hepatocytes contain certain antigens (e.g., albumin); (b) in addition to common liver specific proteins, 6-10

cell deep layers of hepatocytes around the central vein contain antigens not evident in periportal cells; and (c) a subset of pericentral cells contain the above mentioned proteins and glutamine synthetase.

Ontogeny of Antigen Expression

We next examined the presence and distribution of pericentral antigens and glutamine synthetase during fetal and neonatal development. None of the 12 mAbs that stained pericentral antigens (including the eight proven anti-MUP monoclonals) was reactive with fetal or neonatal tissue. At ~15-18 d of postnatal development (about the time of puberty) the pericentral antigen was detectable and the staining distribution remained the same in animals from 3 wk of birth throughout adulthood (Table I). Again, the pericentral pattern was confirmed. These results would be expected for MUPs, as the genes encoding them are not transcribed until puberty (2).

The patterns were very different in the staining of fetal and neonatal tissue with glutamine synthetase antibodies (Fig. 4). Most or all of the hepatocytes in sections in the loosely arranged fetal liver tissue from 15-d and term gestation fetuses were reactive with the glutamine synthetase antibodies (Fig. 4 *b*). After birth this antigen stained less intensely in hepatocytes. Within 3-4 d a more intense staining became evident in the single layer of pericentral cells than other hepatocytes (Fig. 4, *c* and *d*). By 12 d after birth the adult pattern was established (Fig. 4, *e* and *f*). Antibodies to antigens such as albumin, which are known to be expressed in hepatocytes early in fetal life (8), stained fetal cells uniformly, as was the case in adults (Fig. 4 *a*; 42, 55). Thus cells that are already determined to be hepatocytes and are already localized within the liver architecture appear to undergo changes in gene expression to reach the final regional distribution of expression characteristic of the adult.

Liver-specific Antigen Expression in Cultured Cells

It is a common observation that disaggregated adult hepatocytes placed in cell culture with serum containing medium lose their liver specific functions within a few days to a week (6, 11, 12, 30, 40, 44). We tested cultured adult hepatocytes for the presence of pericentral antigens. Approximately one-half of the plated cells were positive for the MUP antigens early in the course of culture (Fig. 5, *c* and *e*). A very few cells were positive for glutamine synthetase (Fig. 5 *f*), and all such cells also expressed MUP. After 1 wk in vitro almost all cells had lost immunoreactivity for these antigens (Table I).

Culturing fetal hepatoblasts proved to give a different and somewhat surprising result. Microscopically the cells plated from 13-d gestation liver buds gradually came to resemble mature adult hepatocytes (Fig. 6), acquiring such morphological characteristics as a dark, grainy cytoplasm, prominent nucleus and nucleoli, with some cells becoming binucleate (16, 63). About 10-20% of the total cells were in these hepatocyte-like clusters and virtually all such cells stained with anti-albumin antibody (Fig. 7 *g*). The cells between clusters were of various types (including fibroblasts, adipocytes, beating heart cells, neuronal-like cells, endothelial cells, etc.) and did not contain immunologically detectable albumin. As we noted above, sections of fetal liver (or of whole fetuses) showed no cells reactive with any of the peri-

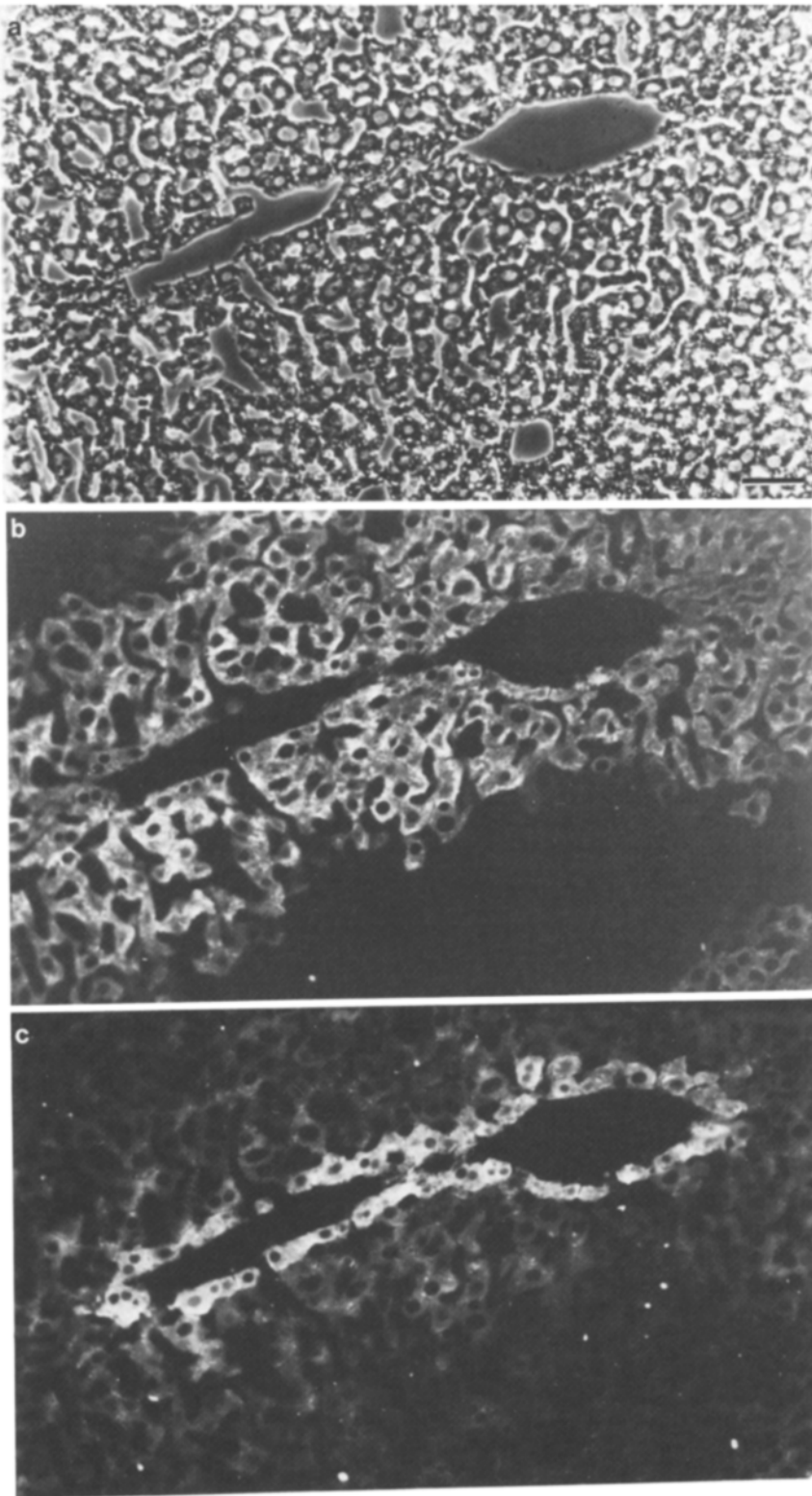


Figure 3. Location of glutamine synthetase in adult mouse liver. A section of adult mouse liver was labeled with antiserum prepared against glutamine synthetase purified from adult rat liver. The single layer of stained cells (*c*) is compared with the more general pericentral staining for MUP mAbs (*b*) in the same section, which is shown in phase (*a*). Bar, 54 μ m.

central antibodies. Cells from the liver of 19-d gestation fetuses (where the liver cells are reasonably well differentiated) or cells from the liver bud of 13-d gestation animals (where undifferentiated hepatoblasts are extirpated along with bits of other surrounding tissues) likewise did not stain with the pericentral antibodies during the first few days of

culture (Table I). Beginning \sim 7–8 d after explanting cells from 13-d gestation fetuses, and increasing with time in culture, cells that were positive for the pericentral antigens began to appear (Table I) in the clusters of differentiated hepatoblasts.

By 12–15 d in culture the number of cells in the hepatocyte-

Table I. Timecourse of Pericentral Antigen Expression In Vivo and In Vitro

Age of animals	Pericentral antigen expression	Days in culture	Pericentral antigen expression in 13-d fetal liver cultures	Pericentral antigen expression in adult hepatocyte cultures
Days of gestation				
13	—	1	—	+++
14		2	—	+++
15		3	—	++
16		4	—	++
17		5	—	+
18		6	±	+
19	—	7	±	—
Birth		8	+	
Days postpartum				
1		9	+	
2		10	++	
3	—	11	++	
7	—	12	+++	
12	—	15	+++	
15	—	20	+++	
18	+	23	+++	
21	++	29	+++	
24	+++	35	+++	
Adult	+++		+++	

—, No antigen expression; +, some antigen expression; ++, moderate antigen expression; +++, full antigen expression.

like clusters that were reactive with the pericentral mAbs had reached a maximum of ~50% and thereafter the fraction of cells that were antigen positive remained at the same level up to 35 d in culture (Table I; Fig. 7, e, h, j, and m). Occasionally small clusters (<25 cells) contained no cells or all cells positive for pericentral antigens. During this time all hepatocytes also remained positive for albumin. In addition to whole FCS (10%), insulin (200 U/l), and hydrocortisone (10 mg/l) were present in the culture medium. Cortisone was necessary for growth and for differentiation of the fetal hepatoblasts. No sex hormones were added to the medium but we note that in adults both male and female cells express MUP, although females produce a smaller amount of these proteins and possibly express a limited set of the collection of MUP genes (13, 15). Without quantitation of the specific MUP proteins and mRNAs we don't know whether the fetal cells express the same MUPs as do adult cells.

The expression of glutamine synthetase in cultured fetal cells was in sharp contrast to the pericentral antigens. After 5 d in culture virtually all the cells in hepatocyte-like clusters became positive for glutamine synthetase and this antigen remained in all hepatocytes for the next 5–10 d (Fig. 7, b and d). Between ~12–20 d in culture, the staining of cells for glutamine synthetase declined considerably so that after ~20 d only a rare cell that was albumin and MUP positive was also glutamine synthetase positive (Fig. 7, k and n). Thus, the cultured fetal cells underwent a program of protein expression similar to the development of liver cells in vivo. This included the continued presence of albumin for up to 35 d in culture, and the early in vitro appearance of glutamine synthetase, followed by its disappearance from the majority of cells with retention of this antigen in only a few cells. With respect to the pericentral antigens, about one-half of the fetal hepatocytes developed an antigen that normally

would not have been produced in these cells until 2–3 wk after birth.

Various hepatoma and liver cell lines have also been tested by immunofluorescence for the presence of the pericentral antigens, including the human hepatoma Hep-G2 (34), rat hepatomas Fao, C2, and Rev7 (53, 60), mouse hepatoma BWJ (50), and mouse fetal liver cell line BNL CL.2 (48). None of these cells have shown any detectable labeling with any of the pericentral mAbs. There is only one hepatoma line (subclone A49 [61], derived from FU5-5 [49, 60]) so far reported to express α 2u-globulin mRNA, after induction with dexamethasone and insulin (61), however our mAb, which reacts with rat liver, does not label these cells.

Discussion

The present results with monoclonal antibodies and with polyclonal antibodies to glutamine synthetase, as well as earlier results with polyclonal antibodies to several liver enzymes (22, 52, 62), point to differentiation within the hepatocyte lineage of at least three patterns of specific antigen distribution. All hepatocytes share certain differentiated functions such as albumin synthesis (42, 55), other hepatocytes in addition express (either predominantly or exclusively), possibly among a range of genes, those for detoxification enzymes and for at least one secreted protein, MUP (2, 52, 62). Finally, a few adult liver cells continue to express glutamine synthetase (22) which is present in most or all fetal hepatocytes. For all of these proteins the presence of the enzyme in a cell suggests that the cell has that particular gene activated; for secreted proteins it is possible that proteins accumulate in cells where the gene is not active. We do not consider this likely for antigens we have examined, however, because expression of both MUP and glutamine synthetase

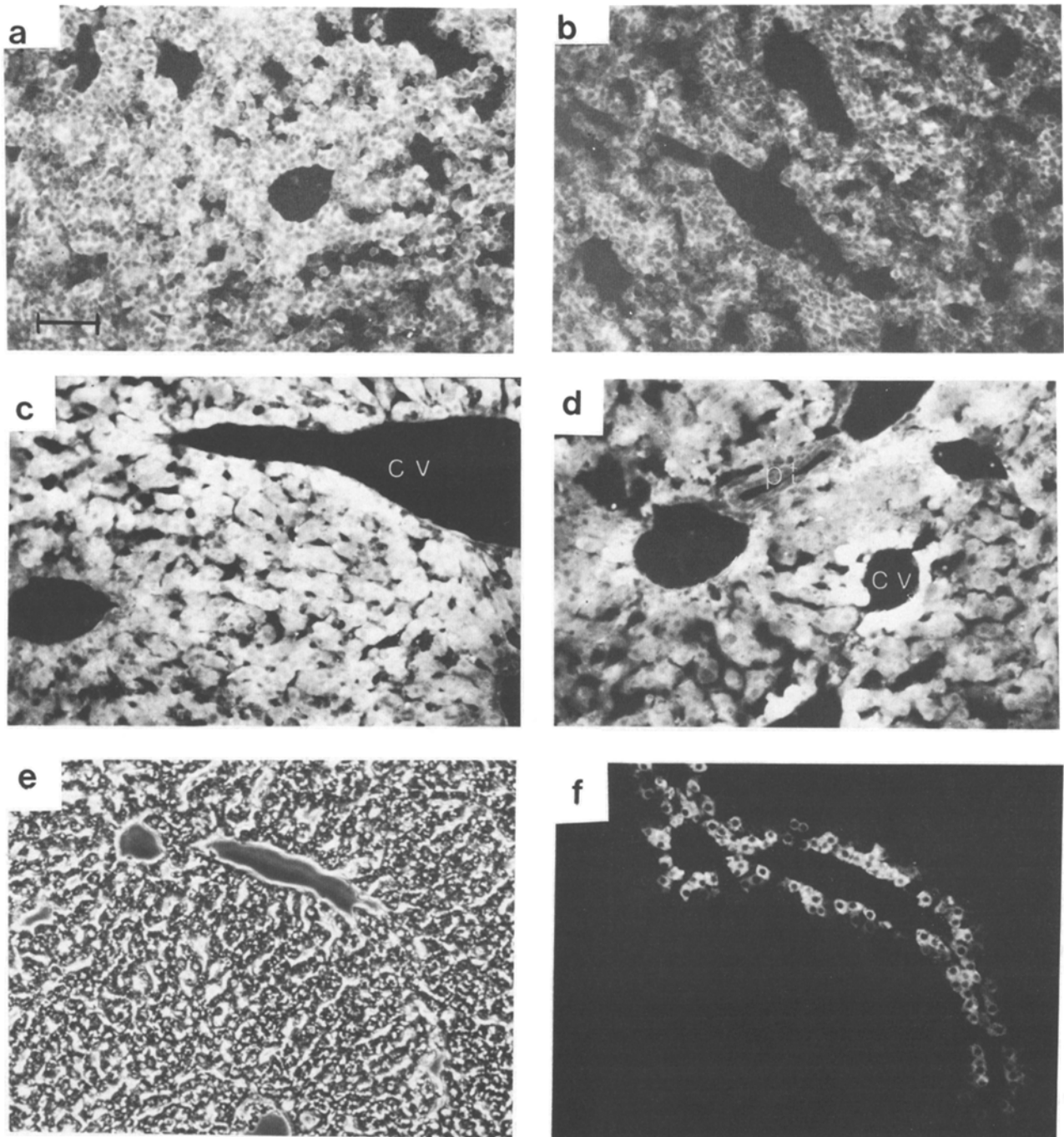


Figure 4. Ontogeny of glutamine synthetase expression. Fetal hepatocytes in sections prepared at 15 d of gestation all show reactivity with both albumin (a) and glutamine synthetase (b) antibodies. After birth the glutamine synthetase declines in the majority of hepatocytes (c, 24-h postpartum; d, 72-h postpartum) such that by 12-d postpartum (e and f) the single cell layer-labeling pattern typical of adult liver is observed. cv, Central vein; pt, portal triad. Bar, 55 μ m.

remains restricted in cultured adult hepatocytes, and most important, for all antigens examined the cells must be permeabilized to react with the antibody. Therefore, the results of the distribution of antigens appear to indicate at least three stable subpopulations of adult hepatocytes with overlapping but different patterns of gene expression, and these three groups of cells are geographically distributed in a particular

fashion: one single cell layer of pericentral cells expresses glutamine synthetase; \sim 6–10 pericentral cell layers express the group of pericentral antigens; and all hepatocytes express albumin. These results correlate well with the notion that the pericentral cells are more highly differentiated, as evidenced by the facts that pericentral hepatocytes stop dividing sooner in neonatal development and are also the least likely to par-

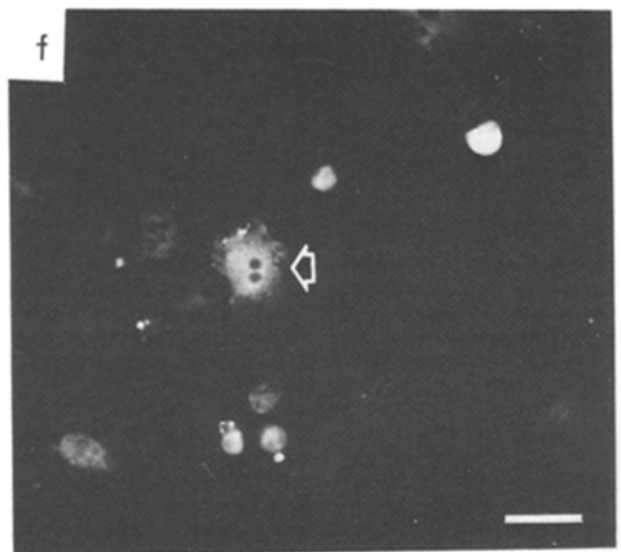
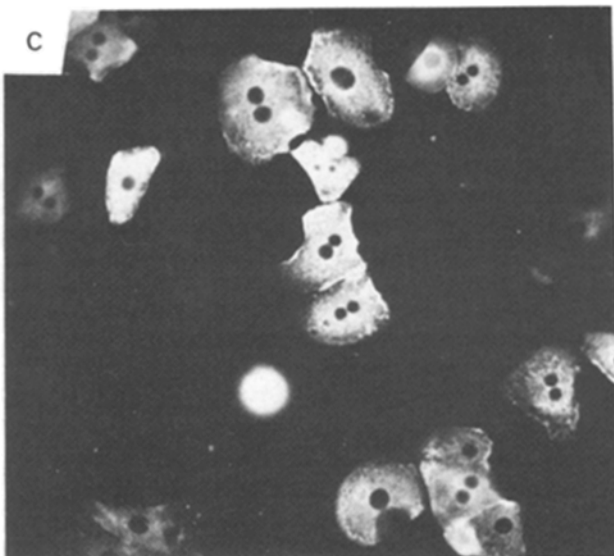
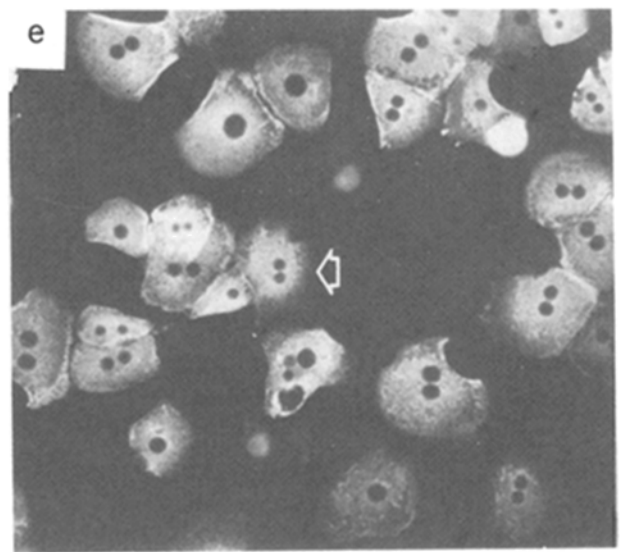
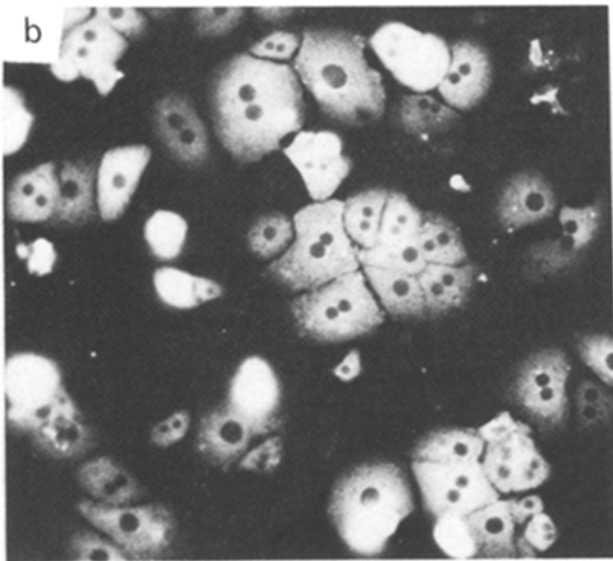
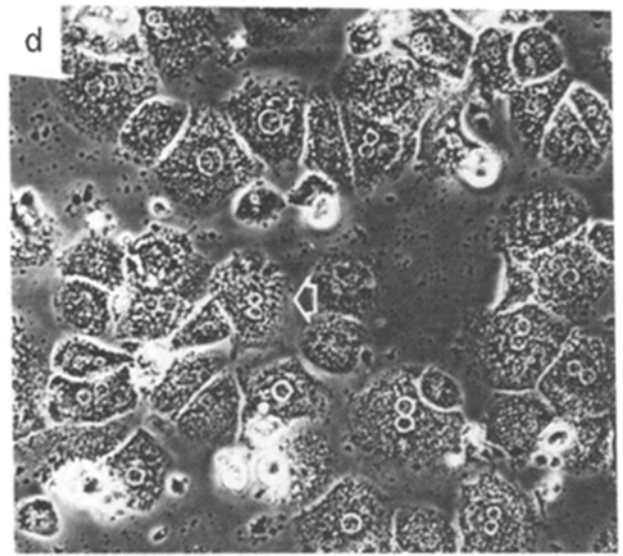
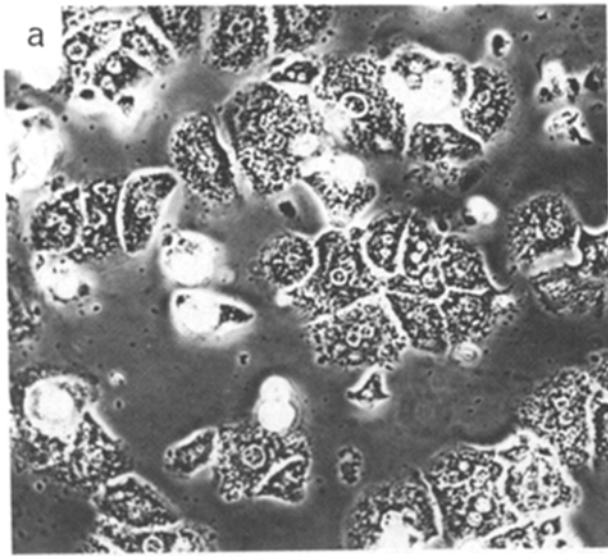


Figure 5. Adult hepatocytes were cultured for ~ 36 h, fixed, and labeled with antibodies. Albumin antibody stained all the hepatocytes (a, phase; b, albumin), whereas only half of these same cells were labeled by MUP mAb (c). The arrow (f) indicates a single cell that is positive for glutamine synthetase, and this same cell is also positive for MUP (e and d, phase). Bar, 55 μm .

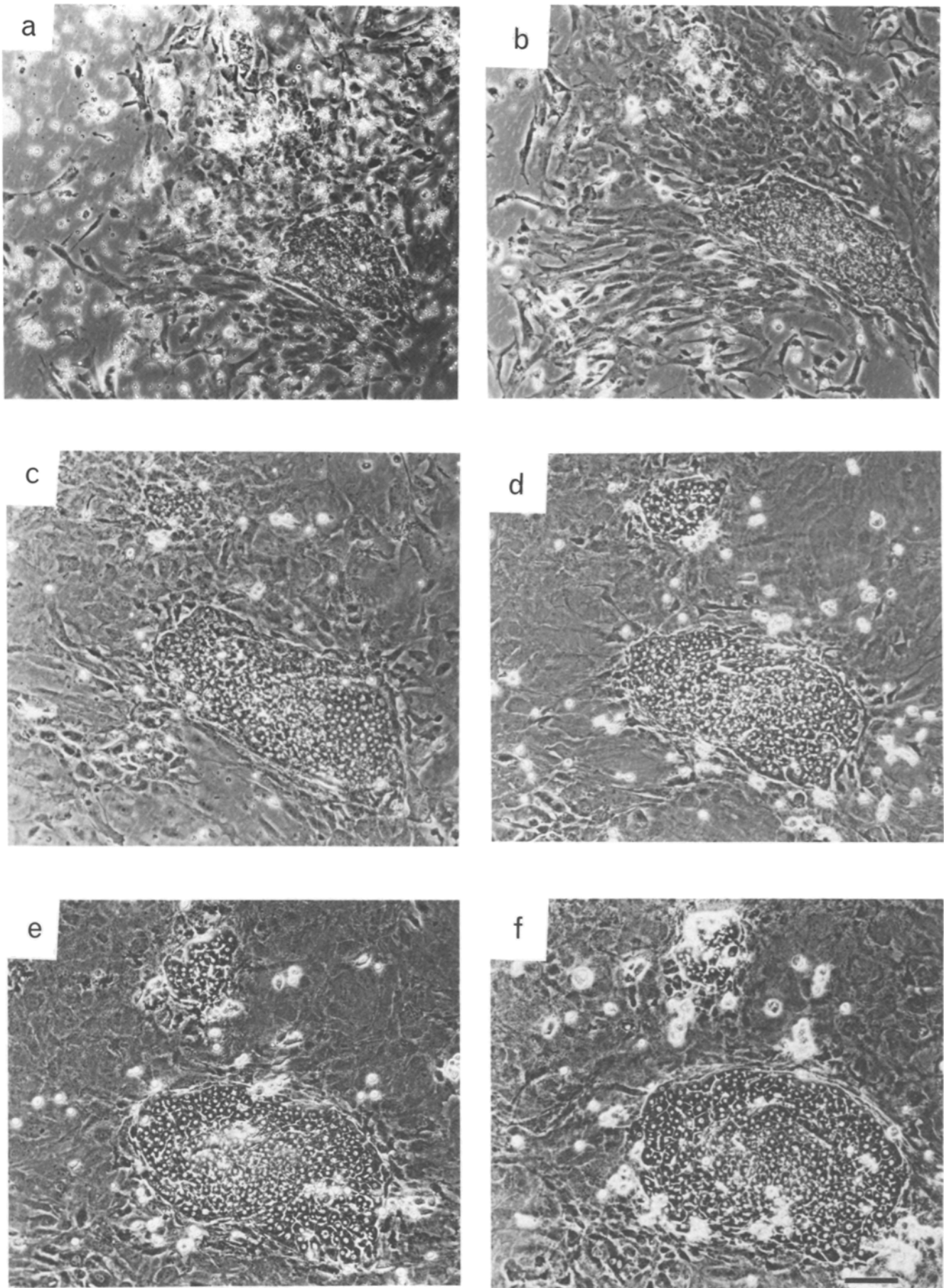
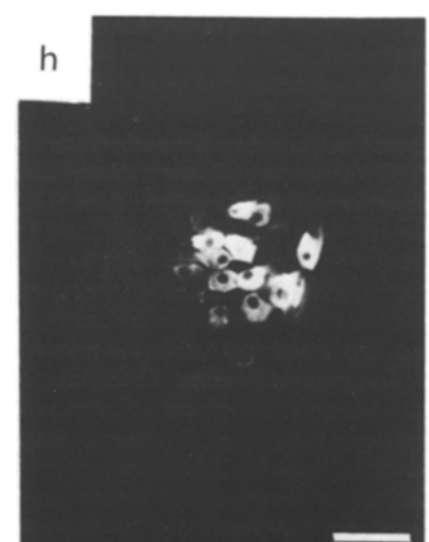
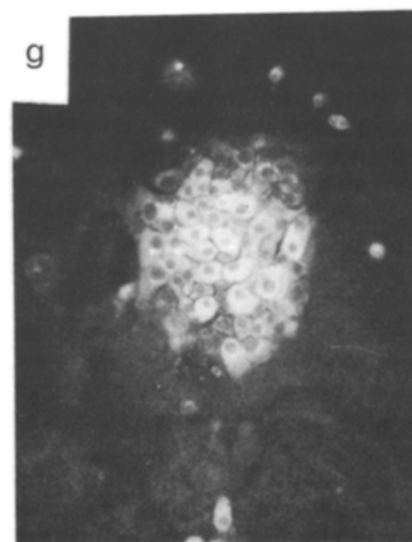
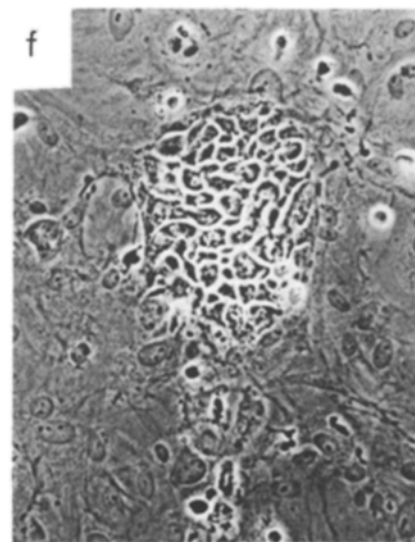
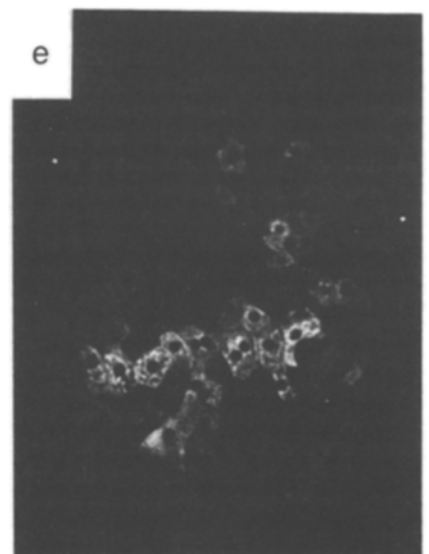
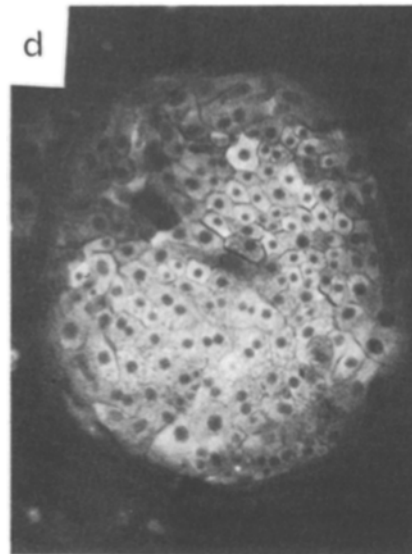
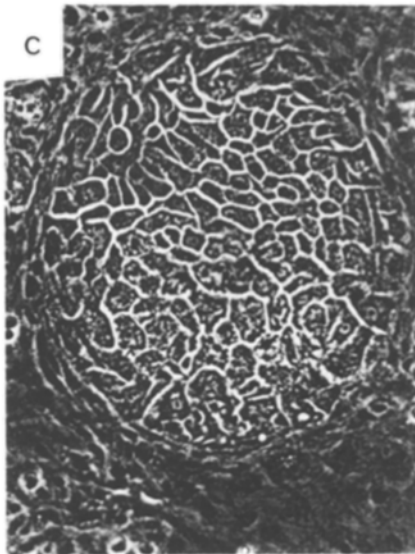
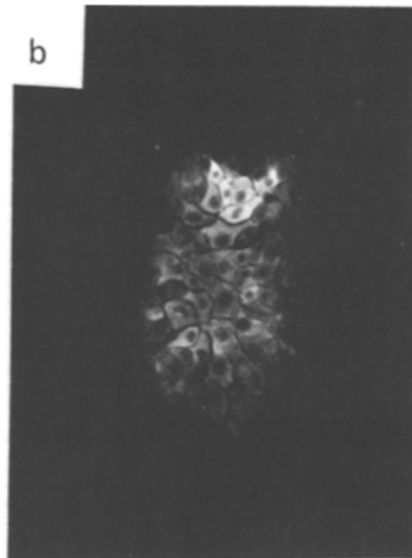


Figure 6. Timecourse of in vitro differentiation of 13-d fetal hepatoblasts. The same two hepatoblast clusters photographed at various times after plating: (a) 1, (b) 2, (c) 5, (d) 8, (e) 10, (f) 13 d of culture. Bar, 111 μm .



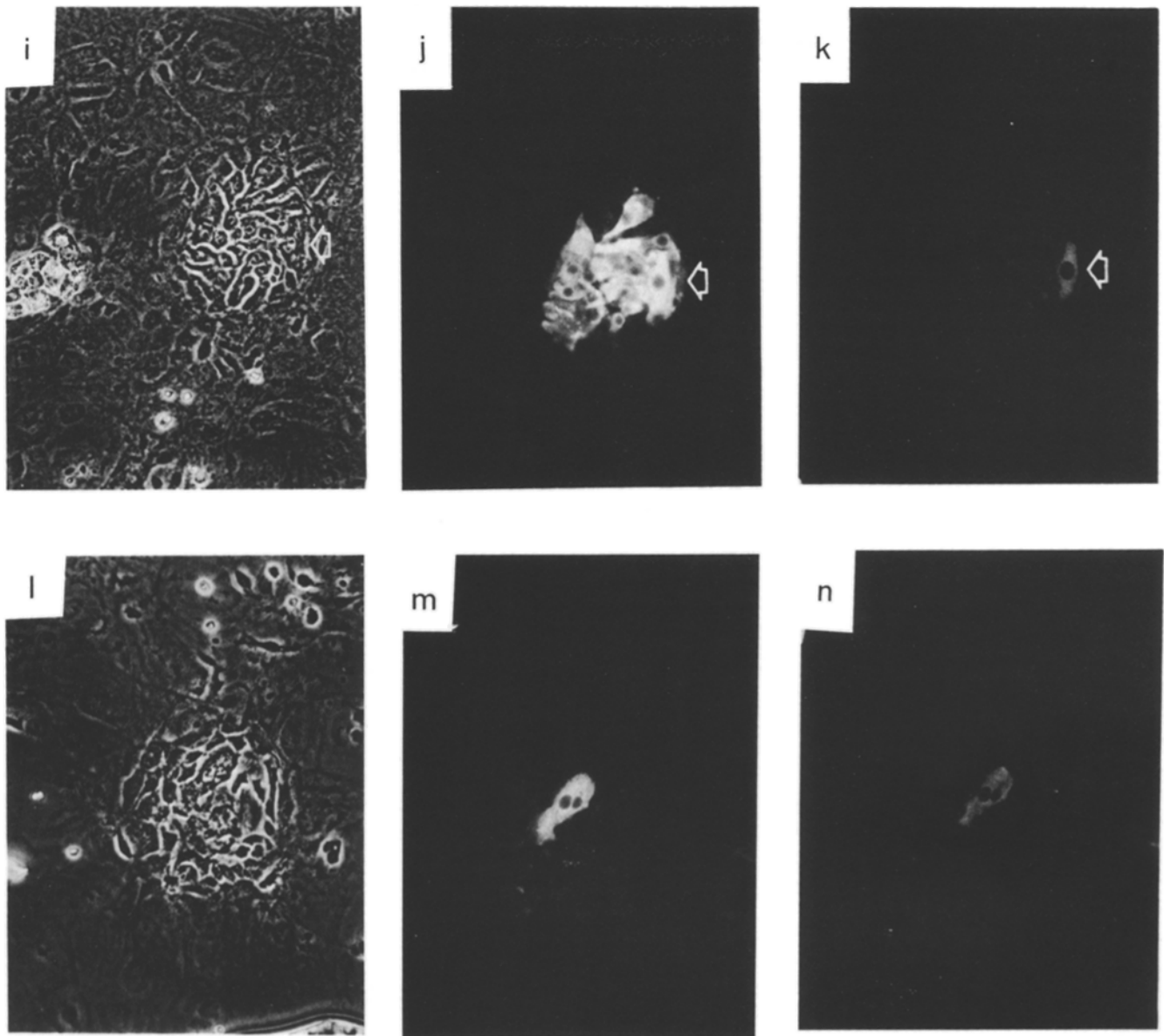


Figure 7. Antigen expression in hepatoblast cultures from 13-d fetuses. Time after plating: 5 d (a) phase and (b) labeled with glutamine synthetase antibody; 9 d (c) phase, (d) glutamine synthetase label, and (e) MUP label; 13 d (f) phase, (g) albumin label, and (h) MUP label. Two examples at 14 d of culture: phase, MUP and glutamine synthetase labeling (i, j, and k, and l, m, and n, respectively). Bar, 55 μ m.

ticipate in compensatory hyperplasia during regeneration (7, 31, 36).

The various types of hepatocyte phenotypes arise by different and interesting ontogenies that are indicative of both positive and negative regulatory events in the expression of these functions. In vivo, albumin expression begins very early in liver organogenesis and continues throughout development in a relatively homogenous distribution in all hepatocytes. Our pericentral antigen(s), including MUP, are not expressed until 2–3-wk postpartum, and arise in their distinctive pericentral location. Glutamine synthetase is apparently expressed initially in the late gestation fetus by all hepatocytes, and becomes restricted to the cells immediately adjacent to the central veins within 1–2 wk after birth. We have reported here results obtained with a novel 13-d gestation hepatoblast culture system, which undergoes both mor-

phological and functional differentiation in vitro in a manner that mimics the in vivo ontogeny. Albumin is expressed throughout the in vitro differentiation process by all the developing hepatoblasts. MUP is first expressed after 1 wk in culture, and from 2 wk on is present in $\sim 1/2$ of all the differentiated hepatoblasts, a proportion equivalent to the in vivo distribution. Glutamine synthetase is expressed within 5 d after plating in virtually all hepatocytes, and declines within 2–3 wk in vitro to be present only in a few cells, all of which also express MUP. This 13-d hepatoblast culture system undergoes a program of differentiation to achieve a distribution of mature hepatocyte phenotypes in which all three subpopulations are represented in appropriate proportions, offering opportunities for the further study of how positional information within the developing liver organizes networks of tissue-specific gene control.

One obvious and important line of research that should be stimulated from these results is the cloning of genes expressed both throughout the liver and in a restricted manner in the liver acinus. The sequences responsible for limiting gene expression should be delineated by introduction of genes into transgenic mice or possibly into differentially functional hepatoma lines. With the aid of adenovirus vectors, which can infect all types of cells, the clearest answers might be forthcoming. We have achieved cell-specific expression of albumin promoter-driven RNA from adenovirus in hepatomas and hepatocytes (3, 17). Thus we might be able to obtain a cell-limited expression of genes such as MUP or glutamine synthetase from adenovirus vectors in either fresh primary hepatocytes or in cultured fetal cells.

Perhaps the most intriguing aspect of the differential expression of genes in various sectors of the liver acinus is the nature of the signals responsible for these differences. It seems most plausible to regard all hepatocytes as members of one initial founder cell lineage that arises in the mouse when primitive endodermal gut cells grow out to contact the precardiac mesenchyme (28, 37, 46). The adult liver is composed of descendants of this group of prehepatic cells. The basis for the observed differences in gene expression could then reside in different signals (for example cell-cell or cell-matrix surface signals) that hepatocytes receive in different regional positions within the liver. This proposal suggests that the matrix produced by endothelial cells of the central vein (or hepatocytes in contact with them) would be different from that produced by the vascular and/or biliary cells in a portal triad (or the hepatocytes surrounding a portal triad). To assign an importance of extracellular structures in the regulation of gene expression seems reasonable based on a number of studies illustrating a role of cell environment on hepatocyte phenotype (28). Our own studies have shown that maintenance of transcriptional signals in explanted adult mouse hepatocytes requires continued cell contact (12).

An alternative suggestion is that the prehepatic founder cells become divided into sublineages or compartments with their similarities as well as some eventual differences in phenotype being preestablished early in development (14, 19-21, 47). In this proposal the hepatoblasts that reside in the pericentral and periportal regions would be destined from early in liver organogenesis to give rise to the differences in phenotypic expression seen in the adult liver. The experiments on cultured cells could shed light on these two possibilities. In 13-d fetal liver cultures, cells differentiate to contain pericentral antigens with a frequency about equal to that seen in adult liver. This result by itself is compatible with either two hepatocyte sublineages existing in 13-d liver buds or with external signals developing so that $\sim 1/2$ of the cells make appropriate contacts to be induced to make the pericentral antigens. Cultures of the fetal liver cells that undergo this differentiatonal step offer the chance with further experiments (for example, antibodies to cell surface structures) to investigate the phenomenon further. The two possibilities need not be considered necessarily mutually exclusive; compartmentalization might establish the basic periportal versus pericentral phenotypes, with expression of glutamine synthetase being regulated by a cell-cell or cell-matrix interaction (for example, by the endothelial cells lining the central veins, as all glutamine synthetase-positive cells are in direct contact with these cells or the matrix they produce).

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