Population Expansion, Clonal Growth, and Specific Differentiation Patterns in Primary Cultures of Hepatocytes Induced by HGF/SF, EGF and TGFα in a Chemically Defined (HGM) Medium

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Abstract. Mature adult parenchymal hepatocytes, typically of restricted capacity to proliferate in culture, can now enter into clonal growth under the influence of hepatocyte growth factor (scatter factor) (HGF/SF), epidermal growth factor (EGF), and transforming growth factor α (TGFα) in the presence of a new chemically defined medium (HGM). The expanding populations of hepatocytes lose expression of hepatocyte specific genes (albumin, cytochrome P450 IIB1), acquire expression of markers expressed by bile duct epithelium (cytokeratin 19), produce TGFα and acidic FGF and assume a very simplified morphologic phenotype by electron microscopy. A major change associated with this transition is the decrease in ratio between transcription factors C/EBPα and C/EBPβ, as well as the emergence in the proliferating hepatocytes of transcription factors AP1, NFκB. The liver associated transcription factors HNF1, HNF3, and HNF4 are preserved throughout this process. After population expansion and clonal growth, the proliferating hepatocytes can return to mature hepatocyte phenotype in the presence of EHS gel (Matrigel). This includes complete restoration of electron microscopic structure and albumin expression. The hepatocyte cultures however can instead be induced to form acinar/ductular structures akin to bile ductules (in the presence of HGF/SF and type I collagen). These transformations affect the entire population of the hepatocytes and occur even when DNA synthesis is inhibited. Similar acinar/ductular structures are seen in embryonic liver when HGF/SF and its receptor are expressed at high levels. These findings strongly support the hypothesis that mature hepatocytes can function as or be a source of bipotential facultative hepatic stem cells (hepatoblasts). These studies also provide evidence for the growth factor and matrix signals that govern these complex phenotypic transitions of facultative stem cells which are crucial for recovery from acute and chronic liver injury.

Several studies in recent years have shown that hepatocyte growth factor, also known as scatter factor (HGF/SF)[1], EGF (epidermal growth factor), and TGFα are the primary mitogens for hepatocytes in culture which stimulate limited hepatocyte DNA synthesis in chemically defined media (for review see Michalopoulos, 1990). These growth factors, first shown to be hepatocyte mitogens in culture, were subsequently found to also play a role in vivo, in liver regeneration after partial hepatectomy. Plasma levels of HGF/SF rise within 30 min after partial hepatectomy (Lindroos et al., 1991). HGF/SF mRNA also increases in mesenchymal cells of regenerating liver 3–24 h after partial hepatectomy (Zarnegar et al., 1991), paralleling a rise of TGFα and acidic FGF production in hepatocytes (Mead and Fausto, 1989; Kan et al., 1989). Injection of HGF/SF, TGFα, or EGF in rats induces DNA synthesis in hepatocytes (Liu et al., 1994; Roos et al., 1995; Bucher et al., 1977; Webber et al., 1994; Ishiki et al., 1992) directly or after pretreatments such as collagenase or "priming" with nutritional manipulations.

Liver regeneration is achieved primarily by cell division of mature adult hepatocytes (Grisham, 1962). These cells (or a fraction of them) have a high capacity for clonal growth, as shown by hepatocyte transplantation experiments in ectopic sites (Jirtle and Michalopoulos, 1982) and in transgenic mouse models (Rhim et al., 1994). It has been shown, however, that when liver is stimulated to regenerate while proliferation of mature hepatocytes is suppressed, facultative stem cells emerge and proliferate (Thorgeirsson et al., 1993; Evarts et al., 1993; Sigal et al.,

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1. *Abbreviations used in this paper*: AP1, activating protein 1; C/EBP: CCAAT Enhancer Binding Protein; CK, cytokeratin; HNF, hepatic nuclear factor; HGF/SF, hepatocyte growth factor; HGM, hepatocyte growth medium; KGF, keratinocyte growth factor; NAD, nicotinamide adenine dinucleotide; NFκB, Nuclear Factor κB; SCF, stem cell factor; uPA, urokinase plasminogen activator.

1992; Dabeva and Shafritz, 1993; Fausto, 1994; Sell, 1994). Several names have been used to describe these cells, of which the term "oval cells" has prevailed for historical reasons. These cells can mature into hepatocytes in defined animal models. Similar cells, however, have been described in end-stage human liver disease such as fulminant hepatic failure. These cells generate hepatocytes or ductular structures composed of cells (ductular hepatocytes) with mixed hepatocyte and bile duct epithelial markers (Gerber et al., 1983; Vandersteenhoven et al., 1990). While the presence of such cells is not doubted, little is known about their origin and about the controls regulating their phenotypic transitions to hepatocytes or ductular cells. The capacity of oval cells to generate ductular cells and hepatocytes parallels the behavior of early embryonic hepatoblasts, which also differentiate toward either hepatocytes or ductular cells (Fausto, 1994).

Despite the high capacity of hepatocytes to proliferate in vivo, directly or via facultative stem cell growth, the conditions that determine their growth potential and their phenotypic transitions are not thoroughly understood due to the hitherto limited potential for hepatocyte growth in primary culture. Typically, under the influence of primary mitogens, hepatocytes in primary culture enter into a limited number of divisions following which they degenerate and die (for review see Michalopoulos et al., 1986). This has been associated with changes in gene expression and changes in specific transcription factor levels (Rana et al., 1994). In other culture systems hepatocyte differentiation is well maintained but cell proliferation is absent or very low (Bucher et al., 1990). The limited capacity of hepatocytes to proliferate in primary culture has hindered applications of hepatocyte cultures to cellular transplantation and gene therapy. Despite more than twenty years of work in this field, there are no culture systems available that allow the population of hepatocytes to expand.

In this report we document conditions that allow hepatocytes to enter into sustained clonal growth resulting in expansion of the cell population. A new chemically defined hepatocyte growth medium (HGM) allows such a growth under the influence of hepatocyte primary mitogens HGF/SF, TGFα, and/or EGF. Under these conditions, hepatocytes undergo multiple proliferative cycles, express altered levels of hepatocyte-associated transcription factors and lose characteristic gene expression markers (albumin, cytochrome IIB1) while expressing aspects of bile duct epithelial phenotype (cytokeratins 14, 19). In the presence of specific matrix components and growth factors, these simplified cells (for which we will use the term proliferating hepatocytes) either revert to a mature hepatocyte phenotype or form acinar structures composed of cells with mixed ductular and hepatocytic appearance. This paper describes the relative effects of specific mitogens and matrix and the roles they play in phenotypic transitions and growth of the proliferating hepatocytes. The results provide new insights on the role of the growth factors, matrix and nutrients as determinants of hepatocyte growth and differentiation. The unexpected phenotypic effects induced by each of these signals provide information about the role of these signals not only in control of growth of hepatocytes but also in determining differentiation of the multiple hepatic lineages (hepatocytic, bile duct epithelial, stem cell, etc.) during liver regeneration and development.

Materials and Methods

Materials

Male Fischer 344 rats from Charles River were used for all the experiments involving hepatocyte isolation. EGF was obtained from Collaborative Research (Waltham, MA). [^{3}H]Thymidine was obtained from ICN Radiochemicals (Irvine CA). Collagenase for hepatocyte isolation was obtained from Boehringer, Mannheim (Germany). Vitrogen (from Celtrix Labs., Palo Alto, CA) was used for the construction of the collagen gels. General reagents were obtained from Sigma Chem. Co. (St. Louis, MO). EGF and Matrigel were purchased from Collaborative Research. HGF/SF used for these studies was the Δ5 variant and was kindly donated by Snow Brand Co (Toshigi, Japan). ECL was purchased from Upstate Biotechnology (Lake Placid, NY).

Isolation and Culture of Hepatocytes

Rat hepatocytes were isolated by an adaptation of the calcium two step collagenase perfusion technique as previously described from our laboratory (Kost and Michalopoulos, 1991). Hepatocytes were plated on a single layer of collagen gel (see below) and left to attach for 2 h. Six-well cluster plates (9.8 square centimeters per plate) from Corning (Ithaca, NY) were used. Unless otherwise specified, 10,000 hepatocytes per square centimeter surface were inoculated for all the experiments described. The medium was changed at 2 h after cells were plated and every 48 h thereafter. Thymidine, growth factors, etc., were added at the time of medium change as required.

Human hepatocytes were isolated by an adaptation of the collagenase perfusion technique as described previously from our laboratory (Strom et al., 1982). Cells were cultured as described above for the rat hepatocytes.

Matrix Manipulations. Collagen gels were prepared as we previously described (Michalopoulos and Pitot, 1975). Dry coating of plates with collagen and Matrigel was also done as previously described. Matrigel gels were made by adding 50 μl of Matrigel solution into 0.5 ml of medium directly on top of attached cells.

DNA synthesis was measured by uptake of tritiated thymidine into TCA precipitable material as previously used in our laboratory (Kost and Michalopoulos, 1991). Collagen gels, where necessary, were digested with 2 mg of collagenase per ml of MEM medium. Incubation was done for 30 min at 37°C. The digested gels were treated with NaOH followed by TCA to precipitate DNA, RNA, and proteins as previously described (Kost and Michalopoulos, 1991).

Composition of the HGM Medium

DMEM, Hepes, glutamine, and antibiotics were purchased from GIBCO/BRL (Gaithersburg, MD). ITS mixture (Insulin, Transferrin, Selenium) was purchased from Boehringer Mannheim. All other additives were cell culture grade (Sigma). Unless otherwise indicated for specific experiments, the basal HGM medium consisted of DMEM supplemented with: purified bovine albumin 2.0 gm/L, glucose 2.0 gm/L, galactose 2.0 gm/L, ornithine 0.1 gm/L, proline 0.030 gm/L, nicotinamide 0.610 gm/L, $ZnCl_2$ 0.544 mg/L, $ZnSO_4$:$7H_2O$ 0.750 mg/L, $CuSO_4$:$5H_2O$ 0.20 mg/L, $MnSO_4$ 0.025 mg/L, glutamine 5.0 mM, ITS 1.0 gm/L, (rh-insulin 5.0 mg/L, human transferrin 5.0 mg/L [30% diferric iron saturated], selenium 5.0 μg/L), and dexamethasone 10^{-7} M. Penicillin and streptomycin were added to the basal HGM at 100 mg/L and 100 mg/L, respectively. The mixed basal HGM was sterilized by filtration through a 0.22-μm low protein-binding filter system, stored at 4°C, and used within 4 wk. The growth factors, as required, were added to HGM fresh at the specified concentrations every time the medium was changed.

Retroviral Transfection and Assessment of Clonal Expansion

Hepatocytes were initially plated at 10^4/cm^2 and grown in HGM supplemented with HGF/SF (40 ng/ml) and EGF (20 ng/ml). After 68 h the media was replaced with supernatant from CRΨP-packaged, replication-deficient, amphotropic retrovirus (MFG, ~5×10^5 units per ml) containing the *E. coli* β-galactosidase gene under an LTR promoter as previously

described (Zitvogel et al., 1994). Polybrene was added at 2 μg/ml. Supernatant was replaced after 18 h with HGM (with EGF 20 ng/ml, HGF/SF 40 ng/ml). The brief exposure with the virus containing supernatant did not have an adverse effect on hepatocyte survival or proliferation. At indicated times, cells were fixed with 0.5% glutaraldehyde in PBS for 10 min and developed with X-Gal substrate at 37°C for 16 h. Transduced cells expressing the *E. coli* gene stained blue as shown. Appropriate controls for each component were negative for X-Gal staining.

Transmission Electron Microscopy

Samples for transmission electron microscopy (TEM) were fixed on the culture plates for 1–1.5 h in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2% formaldehyde. The plates were then rinsed 2× with 0.1 M sodium cacodylate buffer (pH 7.4) and 2× with 0.1 M sodium cacodylate buffer containing 5% sucrose (pH 7.4). They were held in the sucrose buffer for 1–7 d, rinsed 2× with 0.1 M sodium cacodylate buffer (pH 7.4), and then postfixed for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer. The plates were then rinsed again in buffer, and the fixed and processed collagen gels were cut in strips with a razor blade. The strips were transferred to glass specimen vials, dehydrated through a graded series of ethanol (25–100%) and two propylene oxide changes, and infiltrated with Epon-Araldite resin. Several changes of resin were made over 2 d, as the collagen gels tended to hold the propylene oxide. The collagen strips were flat-embedded and cured overnight at 60°C.

Nuclear Extract Preparation

Nuclear extracts were prepared as described (Chiles et al., 1991; Dignam et al., 1983; Wen and Locker, 1994) with several modifications. All cell culture preparations were based on initial plating of 6×10^6 cells in 100-mm plates. Cells were scraped from the plates; washed in 40 mM Tris, pH 7.6, 14 mM NaCl, 1 mM EDTA; resuspended in 2 ml of hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 1 mM DTT, 1 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin, and antipain, plus 1% nonfat dry milk [Tian and Schibler, 1991]); incubated for 10 min at 4°C; and given 60–100 strokes in a Dounce homogenizer. Homogenates were examined under a microscope to control breakage. Released nuclei were pelleted by centrifugation at 800 *g* for 5 min, washed, and extracted in 50 μl of hypertonic extraction buffer (30 mM Hepes, pH 7.9, 25% glycerol, 450 mM NaCl, 0.3 mM EDTA, 6 mM DTT, 12 mM $MgCl_2$, 1 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin, and antipain) for 45 min. After centrifugation at 40,000 *g* for 30 min, the supernatant was recovered and dialyzed for 2 h against the same solution, but containing 150 mM NaCl. Protein concentration was determined with the BioRad protein assay kit (Bradford, 1976). Preparations yielded from 60 to 130 μg protein.

Gel Shift Assays

Single stranded oligonucleotides (Table II) were annealed by incubation at 65°C for 15 min followed by slow cooling to room temperature. Labeling was performed by incubating the double–stranded oligonucleotides with 2 U Klenow DNA polymerase I, 20 μCi each of [^{32}P]dCTP and α-[^{32}P]dATP (each 650 Ci/mmole), in 50 nM dGTP, 50 nM dTTP, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT at 37°C for 60 min. After phenol extraction, the unincorporated nucleotides were removed using a G-50 Sephadex mini column. Specific activities were $\sim 5 \times 10^7$ counts/min/μg. The binding reactions (10 μl) contained 2 ng of double-stranded oligonucleotide probe, 5 μg poly(dI-dC):poly(dI-dC), and 2.1 (AP1, NF1) or 4.2 μg (C/EBP, HNF1, HNF3, HNF4, NFκB) nuclear extract protein in 25 mM Hepes, pH 7.9, 100 mM NaCl, 9 mM $MgCl_2$, 0.25 mM EDTA, 18% glycerol, 5 mM DTT, 0.75 mM PMSF, and 0.75 μg/ml each leupeptin, antipain, and aprotinin. After a 20-min incubation at room temperature, the mixture was electrophoresed through a 6% polyacrylamide gel in 0.5 × TBE running buffer for 1.5 h. For supershifts, 2 μl of antibody to C/EBPα or C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction after 20 min, which was incubated for an additional 1 h at room temperature before electrophoresis. Gels were dried and autoradiographed.

Analysis of Gene Expression by Northern Blots

Extraction of Total RNA and mRNA from Cultures. Total RNA was extracted from unwashed cell cultures using 2.0 ml of RNAzol B (Cinna/Bio TECX Laboratories, Houston, TX) per well and purified per the manufacturer's guidelines. RNA concentration and purity were determined by routine spectrophotometry. Size separation of 20 μg RNA per lane was completed on denaturing 1% agarose gels and transferred to nylon membranes (Amersham, Arlington Heights, IL) by the capillary method. After cross-linking under UV light, membranes were hybridized overnight with specific cDNAs (as indicated in the figures) that had been labeled with [α-^{32}P]dCTP using an Amersham random primer kit. Membranes were subsequently washed under high stringency conditions and exposed to R film (Kodak, NY) for 1–3 d. Quantification of the RNA hybridization bands was performed by laser densitometry.

Sources of cDNA Probes

cDNA probes used to study gene expression were obtained from the following sources: Cytokeratin 8 from Dr. Norman Marceau (Laval University, Quebec City, Canada); Cytokeratin 14 from Dr. Dennis Roop (Baylor College of Medicine, Houston, TX); Cytokeratin 18 from Dr. Robert Oshima (La Jolla Cancer Research Foundation, San Diego, CA); Cytokeratin 19 from Dr. André Royal (University of Montreal, Montreal, Canada); TGFα (rat) originated from Dr. David Lee (University of North Carolina at Chapel Hill); EGF-R (rat) originated from Dr. Sheldon Earp (University of North Carolina at Chapel Hill); aFGF from Amer. Type Culture Collection (catalog No. 78222); aFGF-R from ATCC, Rockville, MD (catalog No. 65796); uPA originated from Dr. Jay Degen (University of Cincinnati, Cincinnati, OH); cytochrome IIB1 from Dr. Steve Strom (co-author); cDNAs for albumin, α fetoprotein, and transcription factor analysis were generated by Dr. Joe Locker (co-author).

In situ Hybridization

In situ hybridizations were performed as described (Shiratori et al., 1995). HGF/SF cDNA and c-MET cDNA with flanking T3 and SP6 RNA polymerase promoters were prepared by RNA-polymerase chain reaction (Frohman, 1990), using rat lung poly(A)$^+$ RNA and sense strand primers (HGF/SF: nucleotides 282-303 [Tashiro et al., 1990]); c-MET: nucleotides 2913-2932 [Chan et al., 1988]) and antisense strand primers (HGF/SF: nucleotides 649-670; and c-MET: nucleotides 3362-3383). ^{33}P-labeled antisense and sense riboprobes were synthesized using SP6 or T3 RNA polymerases and the Riboprobe Gemini Core System II transcription kit from Promega (Madison, WI). Embryos were collected at 16 d of gestation from timed-pregnant rats. The embryos were fixed in 4% formaldehyde. 10-μm-thick sections were cut and placed on aminopropyltriethoxysilane-coated slides. The slides were rinsed in PBS, treated with proteinase K (1 μg/ml), and acetylated with acetic anhydride (0.25%, vol/vol, in 0.1 M triethanolamine, pH 8).

Hybridization with sense and antisense probes, washing of the slides, and emulsion-autoradiography were performed as described (McLaughlin and Margolskee, 1993).

Results

Role of Media Components and Matrix Substrates on Cell Proliferation

The full description of the HGM medium is given in the Materials and Methods. To evaluate the relative importance of the different media components several experiments were performed whose results are shown in Table I (*A*, *B*, and *C*). The components glucose, albumin, dexamethasone, transferrin and selenium, nicotinamide, and trace elements were individually subtracted from the full medium composition (Table I *A*). The total DNA per culture after 14 d of growth is shown. Removal of dexamethasone had the most dramatic effect, followed by removal of nicotinamide. In Table I *B*, growth of cells achieved by day 14 is compared between HGM medium containing diferric transferrin (iron saturated) vs iron unsaturated transferrin. Addition of iron containing diferric transferrin (30% saturation) was much more effective in promoting growth. Addition of elemental iron ($FeSO_4$, 0.1 μM) to the unsaturated transferrin failed to overcome the difference. Table I

Table I.

A. Effect of removal of specific HGM components on hepatocyte growth	
Zero time DNA	13.90 ± 3.60
DNA at day 14 in HGM (with HGF/SF and EGF) supplemented with:	
+All components	84.90 ± 0.50
−Glucose	69.80 ± 2.90
−Albumin	68.70 ± 0.50
−Dexamethasone	13.70 ± 0.20
−(Transferrin and selenium)	63.10 ± 2.00
−Nicotinamide	35.20 ± 1.70
−Trace elements	65.00 ± 2.40
−All components	20.20 ± 4.30
B. Effects of iron and transferrin on hepatocyte growth	
Zero time DNA	9.21 ± 1.01
Diferric transferrin (iron saturated)	70.15 ± 1.21
Diferric transferrin plus added iron (5 μM)	1.83 ± 0.41
Iron poor transferrin	24.84 ± 4.30
Iron poor transferrin with added iron	1.10 ± 0.90
C. Effect of removal of glucose, ornithine, or galactose	
Zero time	11.0 ± 0.4
Control (Gluc.+, Orn.+, Gal.+)	59.0 ± 2.5
Gluc.−,Orn.−,Gal.−	10.6 ± 0.8
Gluc.−,Orn.+,Gal.−	14.4 ± 0.9
Gluc.−,Orn.−,Gal.+	44.2 ± 6.0
Gluc.+,Orn.−,Gal.−	59.0 ± 4.0
Gluc.+,Orn.+,Gal.−	62.7 ± 1.4
Gluc.+,Orn.−,Gal.+	63.6 ± 0.3

The indicated HGM components were removed and hepatocytes grew in the modified media for 14 d. The total DNA per culture in micrograms was measured at day 14 to evaluate cell growth. Time zero was the sample of hepatocyte suspension that was inoculated into the plate immediately after cell isolation. The data are expressed as mean ± standard error of three separate plates.

C provides information on the relative effects of glucose, galactose, and ornithine. All three of these components are potential sources of energy to the cells. Complete cessation of growth was noticed when all three were removed. Addition of glucose alone restored most of the response whereas addition of galactose alone was less effective. Ornithine alone had minimal effect. Concentrations of 2 g per liter each of albumin and glucose were found to be optimal though the effects were not statistically different than 1 or 3 g per liter in each case. The effect of complete removal of these components was shown in Table I *A*.

Several matrix substrates promoted cell growth in this system. Dry coating with collagens type IV (mouse), type I (bovine), fibronectin, and laminin were equally effective in promoting cell growth as assayed by measurement of total DNA per culture at day 14. Dry coating with ECL (a commercial derivative of EHS gel, UBI) had in some cultures superior effects but the effect was not consistent (data not shown). Coating with type I collagen (Vitrogen commercial preparation) was the standard method used for the subsequent experiments unless otherwise specified. The effect of matrix gels in promoting specific phenotypic conversions in these cultures is discussed below.

Hepatocytes Enter Diffusely into Proliferation under the Influence of HGF/SF, EGF, and TGFα

Fig. 1 (*A* and *B*) shows the uptake of thymidine per μg DNA, as well as the BRdU nuclear labeling index at different days in culture in cells growing in the presence of HGF/SF and EGF. Most of the proliferation occurs at days 5–12. By day 15 the cultures were confluent and DNA synthesis slowed down. The high nuclear labeling index during the times of sustained proliferation documents that the proliferating cells are not a minor contaminant population but they derive directly from the mature parenchymal hepatocytes (see Discussion).

The growth factors HGF/SF, EGF, TGFα, KGF, SCF, and aFGF were added individually to HGM medium. Of the growth factors added, only HGF/SF, EGF, and TGFα caused significant cell proliferation, as shown by the total amount of DNA per culture at day 15. Keratinocyte growth factor (KGF), acidic fibroblast growth factor (aFGF), and stem cell factor (SCF) when added alone or in combination had no effect (data not shown). TGFα had a stronger proliferative effect than any of the other mitogens added alone. HGF/SF and EGF together had the strongest proliferative effect altogether than any other single mitogens or combinations. Addition of all the growth factors together had no more effect than the combined HGF/SF and EGF (data not shown). The detailed cell kinetics induced by HGF/SF and EGF alone or in combination are shown in Fig. 1 *C*. The total DNA per culture is shown as a function

Table II. Oligonucleotides Used in the Gel Shift Studies

Factor	Oligonucleotide	Reference
C/EBP	tcgaATCTTACTCAACAT TAGAATGAGTTGTAagct	(Costa et al., 1988; Locker, J., unpublished)
HNF1	tcgaTGTGGTTAATGATCTACAGTTA ACACCAATTACTAGATGTCAATagct	(Brunel et al., 1988)
HNF3	tcgaAGTTGACTAAGTCAATAATCAGAATCAG TCAACTGATTCAGTTATTAGTCTTAGTCagct	(Costa et al., 1989)
HNF4	tcgaGCGCTGGGCAAAGGTCACCTGC CGCGACCCGTTTCCAGTGGACGagct	(Sladek, 1990)
AP1	tcgaAGGGGCCATGTGACTCATTACACCAG TCCCCGGTACACTGAGTAATGTGGTCagct	(Xanthopoulos, 1991)
NFκB	tcgaTCGAGGGCTGGGGATTCCCCATCTC AGCTCCCGACCCCTAAGGGGTAGAGagct	(Tewari, 1992)
NF1	tcgaATTTTGGCTTGAAGCCAATATG TAAAACCGAACTTCGGTTATACagct	(Nakabayashi et al., 1991)

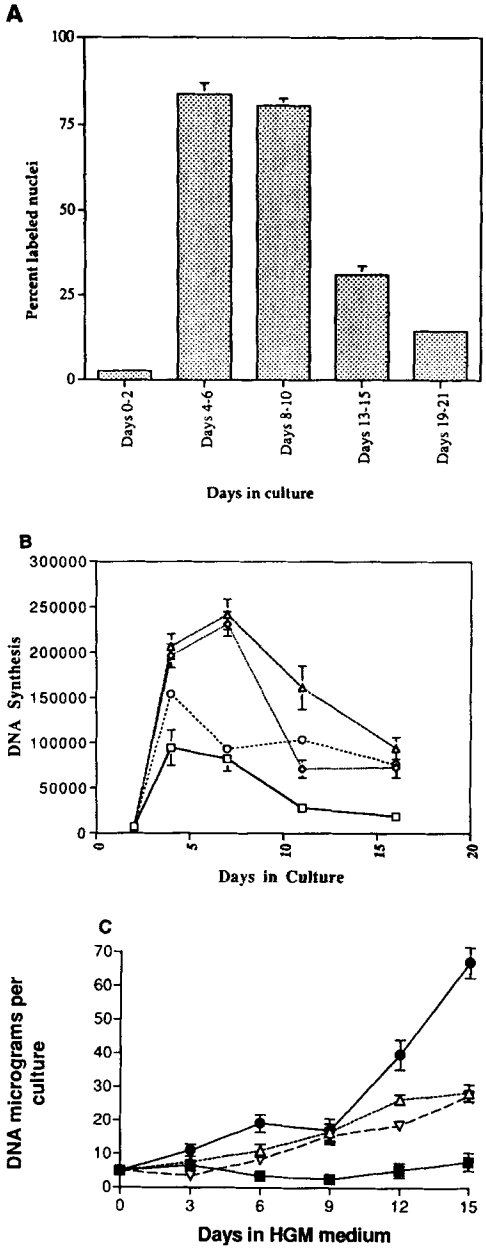


Figure 1. (*A*) Labeling index (BRdU) of proliferating cultures at different times after hepatocyte isolation. Cells were exposed to HGF/SF and EGF in HGM medium. (*B*) Incorporation of [^{3}H]thymidine into DNA in cultures at different times after hepatocyte isolation. Cells were exposed to HGF/SF and EGF in HGM medium. Symbols: Control: □; HGF/SF: ◇; EGF: ○; HGF/SF+EGF: △. (*C*) Amount of DNA per plate at different days in culture as indicated. Cells were kept in HGM and exposed to the indicated growth factors at the concentrations stated in Materials and Methods. Control: □; HGF/SF: △; EGF: ▽; HGF/SF+EGF: ●. All points represent the mean and standard error of at least three separate cultures.

of the time in culture. The largest amount of accumulated DNA at day 15 was seen with the combination of HGF/SF and EGF. The DNA per culture at day 15 was 12 times that at time 0, reflecting the increase in cell number. HGF/SF and EGF were equally potent. Again TGFα alone was more mitogenic than either HGF/SF or EGF alone. Whereas, however, HGF/SF and EGF synergized, no such synergism was seen with HGF/SF and TGFα (data not shown).

Phenotypic Changes of Hepatocytes during Proliferation

The morphology of the proliferating cells varied at different times after stimulation of cell proliferation. From a normal hepatocyte morphology at day 1 (Fig. 2 *A*), the proliferating cells in the first 4 d acquired long projections assuming the phenotype typically described as due to the "scattering" effect of HGF/SF on hepatocytes (Fig. 2 *B*) (Michalopoulos et al., 1993). Between days 6 and 8 (Fig. 3 *A*) the proliferating cells lost most of their cytoplasmic granules, the nuclei became less prominent, the projections diminished and the cells began to grow as monolayer patches. Eventually these patches merged as the cells continued to grow to form a continuous monolayer (Fig. 2 *C*). Examination by electron microscopy (Fig. 2 *D*, and *E*) showed that most of the features typical of mature hepatocytes were missing. By day 15 there were no lamellae of endoplasmic reticulum wrapping around mitochondria and there are no glycogen rosettes or peroxisomes. Bile canaliculi are absent. There is a prominent increase in bundles of keratin intermediate filaments. The nuclei were angular with very prominent nucleoli. This morphology remained stable for several months. The overall size of the cells became smaller with increasing days in culture as the cells continued to proliferate. No noticeable differences in electron microscopic morphology or percent of nuclear labeling with BRdU were noticed based on cell size.

Clonal growth of the proliferating hepatocytes is demonstrated in Fig. 2, *F* and *G*. Hepatocytes were transfected at day three in culture with a replication-deficient retrovirus containing the lac-Z gene under the influence of a viral LTR and stained for expression of β-galactosidase. Mostly single cells were stained positive at day four in culture (1 d after transfection). On the other hand, staining at day 10 and continuing through day 28 showed predominantly patches of hepatocytes, suggesting clonal growth of the original (single cell) transfected hepatocyte precursors. The percentage of lac-Z–positive cells (~20%) did not appear to change during culture (data not shown).

Proliferating Hepatocytes Lose Hepatic Differentiation Markers and Produce TGFα and aFGF and Cytokeratin 19

The expression of several specific genes was assayed in proliferating hepatocytes. These included mRNA genes associated with hepatocyte differentiation (albumin, cytochrome IIB1), genes encoding cytokeratin markers (cytokeratins 14, 18, and 19) or related to hepatocyte growth (urokinase [uPA], HGF/SF and its receptor c-met, EGF and TGFα and their receptor, acidic FGF and its receptor, and TGFβ1). These genes were studied by Northern blot analysis of RNA from cultures grown in the presence of either combined HGF/SF and EGF or with TGFα alone. The results are shown in Fig. 3. Total RNA was isolated from cultures at days 0, 6, 10, 15, and 21. No expression of

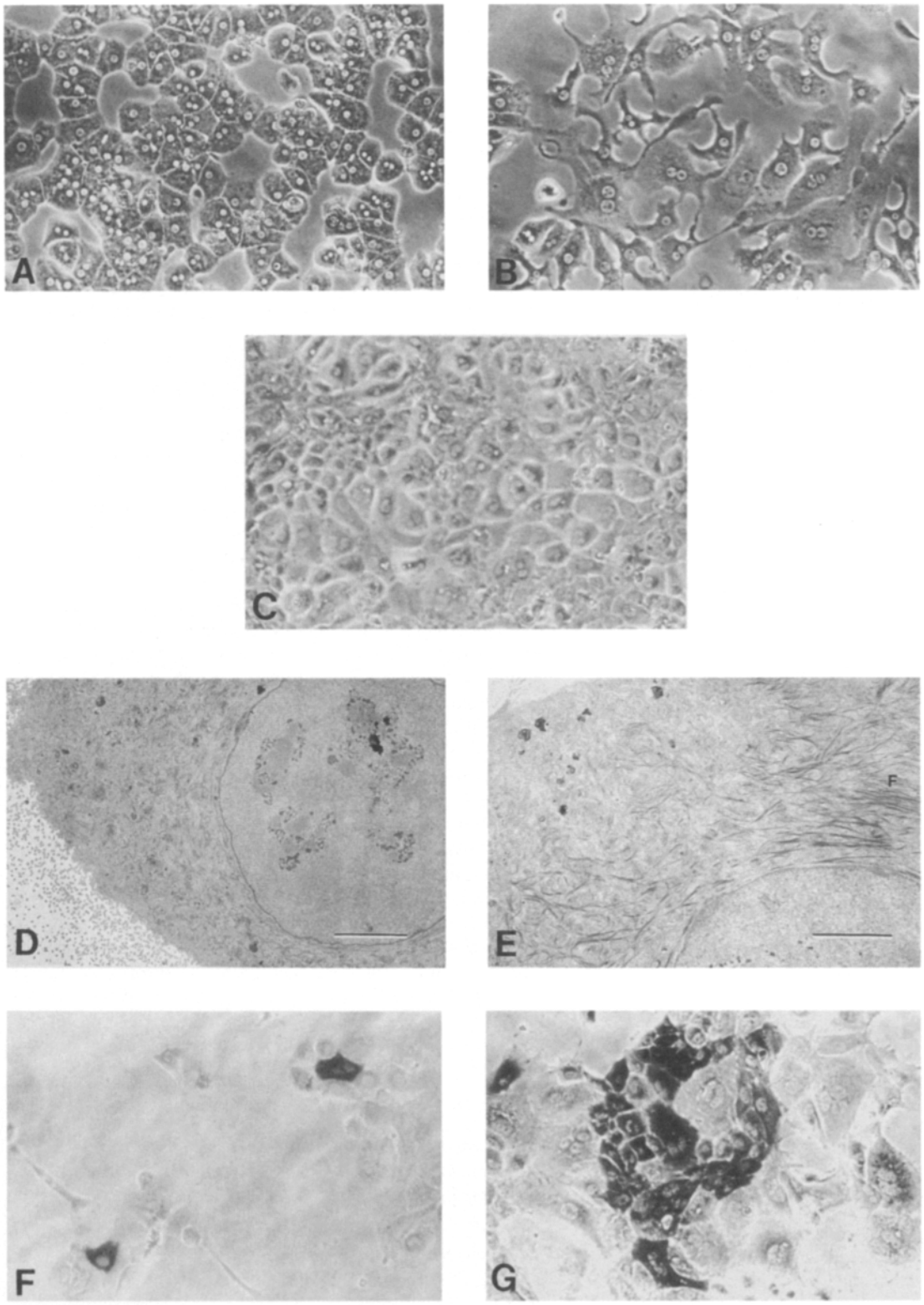


Figure 2. (*A*) Hepatocytes in HGM medium with HGF/SF and EGF at day 1 after isolation showing typical nonproliferating hepatocyte morphology. (*B*) Hepatocytes at day 4 in culture showing the typical scattered morphology induced by HGF/SF. (*C*) Cultures at day 15 showing the typical proliferating hepatocyte morphology with clear cytoplasm, small size, and compact monolayer formation. Photomicrographs of cells in Fig. 2, *A*, *B*, and *C* were taken with under phase contrast microscopy, at a magnification of 100. (*D* and *E*) Electron micrographs of cells at day 15 in culture, in the presence of HGF/SF and EGF. Note the absence of features typical of hepatocyte cytoplasm (contrast to Fig. 5, *B* and *C*) and the presence of prominent nucleoli, bundles of keratin intermediate filaments (*F*) and sparse mitochondria. (*F* and *G*) Phase contrast photomicrographs of cultures transfected at day 3 with a lac-Z–containing replication defective retrovirus (for details see Materials and Methods). Cells were stained for β-galactosidase expression and photographed at days 1 and 10 after transfection. Mostly single cells are seen at day 1 whereas at day 10 typically patches of multiple positive cells were present. Magnification, 100. Bars: (*D* and *E*) 5 μm.

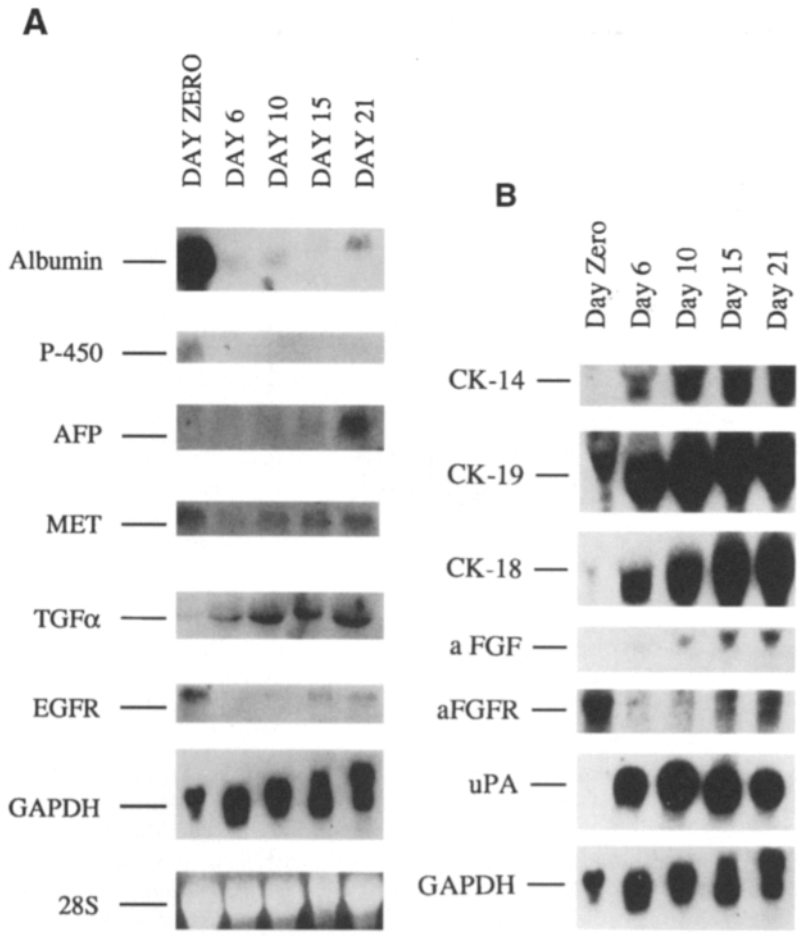


Figure 3. Expression of specific genes at different days in cultures maintained in HGM in the presence of HGF/S and EGF. The expression of the specific genes indicated was assessed by Northern gels as indicated in Materials and Methods. GAPDH expression and the intensity of the 28S RNA after ethidium bromide staining were used as internal controls to compare the amount of loaded RNA between lanes.

HGF/SF or TGFβ1 was seen at any of the time points examined.

Albumin and cytochrome IIB1 mRNA were present at time zero and subsequently decreased. Albumin mRNA appeared again at day 21 at which time mRNA for α fetoprotein was also detected. mRNA for cytokeratins 14, 18, and 19 increased through culture. There was a steady increase in mRNA for aFGF and TGFα. The mRNA of receptors for HGF/SF and aFGF remained present throughout the culture time. The EGF receptor mRNA declined from day zero but remained expressed. GAPDH mRNA expression was used as a reference "housekeeping" gene. Dramatic increases were noted in expression of urokinase as well as cytokeratins 14 and 19. Some differences from the above pattern were seen in the cultures that, instead of HGF/SF and EGF, were maintained in the presence of TGFα. In these cultures albumin expression and expression of the HGF/SF receptor were better preserved whereas AFP appeared earlier. Despite the observed differences in gene expression patterns no morphologic differences were seen between cells growing in the presence of TGFα of HGF/SF plus EGF.

Proliferating Hepatocytes Lose C/EBPα but Express Increased Levels of AP1 and NFκB While Maintaining Other Hepatocyte Transcription Factors

To evaluate the controls which maintain the hepatocytic phenotype, transcription factors were analyzed in nuclear extracts prepared from cultures maintained in HGF/SF and EGF (for details see Materials and Methods). Gel shift assays (Fig. 4) were performed to assess hepatocyte-enriched transcription factors C/EBPα, HNF1, HNF3, and HNF4; general factors associated with proliferation, such as AP1 and NFκB; and NF1, a family of housekeeping factors. Extracts from 1-, 5-, 11-, 15-, and 20-d cultures were compared to freshly isolated hepatocytes. The latter did not differ significantly from intact liver (data not illustrated). The day 1 extracts showed a marked alteration of several factors that did not persist and by day 5 a stable pattern was established that persisted through day 20. The day 1 pattern presumably represents a response to hepatocyte isolation and culture; the most important features of this pattern are marked elevation of C/EBPα, C/EBPβ, and AP1, and decrease of HNF4. The day 5 pattern can be considered a transition to the stable pattern, which differed qualitatively from time 0 hepatocytes as follows: (*a*) strong expression of AP1 and NFκB; (*b*) a marked increase in HNF4, mostly attributed to the appearance of a second band just above the usual HNF4 band shift; and (*c*) decrease of C/EBPα after day 1. Other factors showed relatively minor differences from hepatocytes. Because of possible variation in the efficiency of factor extraction in the different preparations, factor levels were compared directly and also normalized to NF1 as a possible unchanging internal control (e.g., compare the HNF1 and NF1 pat-

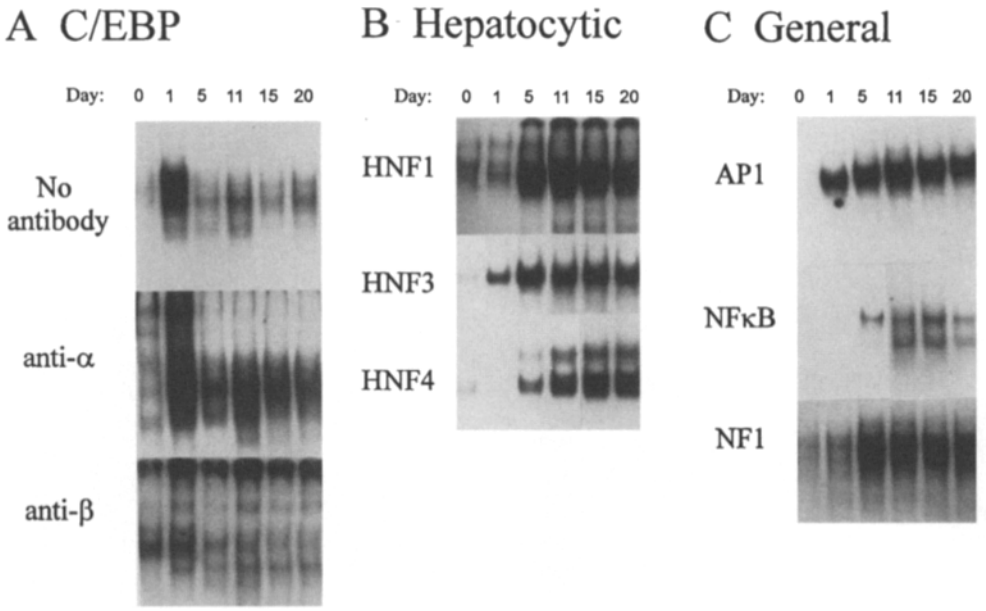


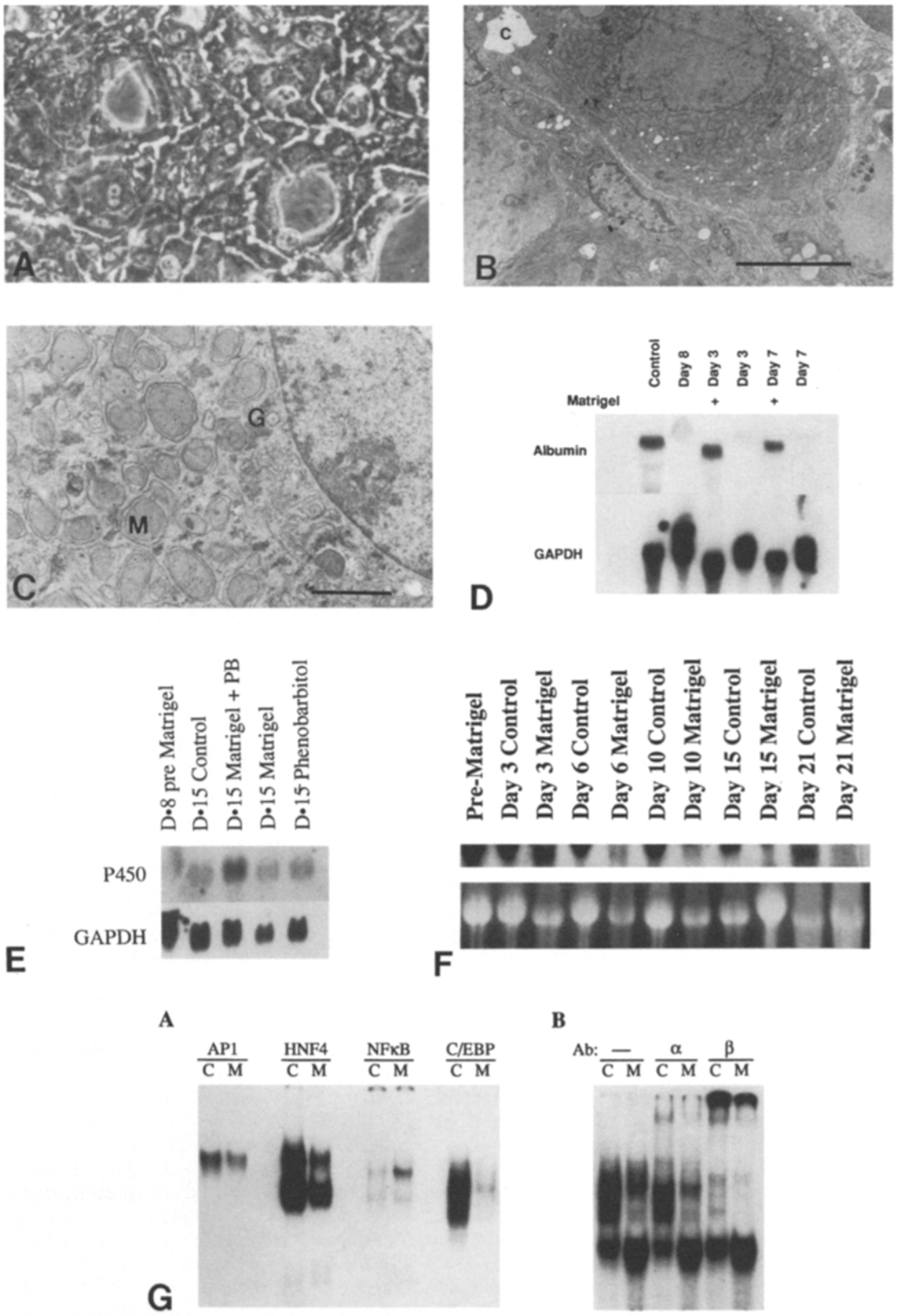
Figure 4. Analysis of transcription factors in proliferating hepatocyte cultures. The illustrated analyses were carried out on a single series of cultures established at the same time and collected as single preparations on days 1, 5, 11, 15, and 20. Day 0 represents the preparation of primary hepatocytes used to establish the cultures. Each individual series used matched concentrations of nuclear extract proteins. (*A*) C/EBP analysis. The panels show a standard gel shift (*upper*) and supershift patterns using antibodies to C/EBPα (*middle*) or C/EBPβ (*lower*). The patterns represent C/EBPα and C/EBPβ homo- and heterodimers, and other less abundant C/EBP family members. The film exposure was reduced in the upper panel. (*B*) Other liver-enriched factors. Standard gel shifts for the HNF1 family (HNF1α and β homo- and heterodimers); HNF3 family (predominantly HNF3β); and HNF4. (*C*) Factors expressed in most cell types. Gel shifts for AP1, NFκB (p65:p50 and p50:p50), and NF1 (multiple factors). For *B* and *C*, exposures were optimized for each individual series.

terns). This normalization did not alter the qualitative description. The stable pattern characteristic of the hepatocytes in HGM medium with HGF and EGF is consistent with a proliferating cell population (elevated AP1 and NFκB) that shows strong expression of the factors associated with the differentiated hepatic phenotype (HNF1, HNF3, HNF4, and C/EBPβ) except for C/EBPα.

Proliferating Hepatocytes Revert to Mature Hepatocytes under the Influence of Matrigel or in the Presence of Nonparenchymal Cells

In confluent cultures maintained longer than 60 d there was focal overgrowth of fibroblast-like cells. Though the nature of these cells is not clear, it has been shown that these cells are derived mostly from hepatic Ito cells (Maher et al., 1988). In those areas, the cytoplasm of the hepatocytes became gradually more granular. Within one to two weeks following this change, typical parenchymal hepatocytes were noted, with granular cytoplasm, prominent bile canaliculi (LeCluyse et al., 1994), bright round nuclei, and prominent nucleoli. We assume that this reversion was dependent on new matrix synthesized by fibroblast-like cells (Maher et al., 1988), although soluble factors elaborated by fibroblast/Ito cells might also contribute. Previous studies (Bissell et al., 1987) have shown that hepatocytes maintained their differentiated phenotype when kept in Matrigel (a commercial derivative of matrix extracted from the EHS mouse sarcoma). This matrix preparation has high laminin content, but it also contains type IV collagen, entactin, decorin, TGFβ1 and glycosaminoglycans, and several growth factors, including $TGF\beta_1$ (Kleinman et al., 1982). When Matrigel was overlaid on cultures at day 8, there was a rapid (within 2 d) appearance of bile canaliculi and organization of the cells along plate structures. The features of these cells are shown in Fig. 5 *A*. By electron microscopy (Fig. 5, *B* and *C*) these cells had typical markers of mature hepatocytes, including wrapping

Figure 5. (*A*) Phase contrast photomicrograph of cultures of proliferating hepatocytes overlaid with Matrigel at day 8 and photographed at day 18 (10 d after overlay of the Matrigel). Note the granular cytoplasm and the appearance of typical bile canaliculi (bright lines between cells) (LeCluyse et al., 1994). Contrast the appearance of these cells with those of Fig. 2 *C*. (*B* and *C*) Low and high power electron photomicrographs of cells at day 18 in culture, 10 d after overlay with Matrigel. Note the presence of typical features of hepatocyte cytoplasm such as lamellae of endoplasmic reticulum wrapping around mitochondria, microbodies with crystalline center, bile canaliculi (*c*) abundant mitochondria (*M*), and (in 5 *c*) glycogen (*G*). Contrast with the cytoplasmic features of the cells in Fig. 2, *D* and *E*. (*D*) Increased expression of albumin mRNA after addition of Matrigel. Albumin mRNA is expressed in control cultures (immediately after isolation from liver by collagenase perfusion and before culture). Expression is minimal at day 8 in culture. At days 3 and 7 after Matrigel addition (lanes marked with +), there was an increase in expression of albumin mRNA. Such increase was not noted in control cultures (lanes of days 3 and 7 not marked with +). (*E*) Induction of cytochrome IIB1 mRNA in cultures treated with Matrigel and exposed to Phenobarbitol (PB). Matrigel was added to the cultures at day 8. PB was added 2 d later (day 10 of culture). The cells were harvested 5 d after addition of PB (day 15 in culture). GAPDH expression was used as internal control for mRNA loading. (*F*) Cytokeratin 19, a bile duct marker expressed by the nondifferentiated proliferating hepatocytes, is suppressed by addition of Matrigel. Matrigel was added at day 8 in culture. Control cultures did not receive Matrigel. CK19 is suppressed by Matrigel while it continues to be expressed by control cultures. (*G*) Matrigel modifies transcription factor expression. *C* designates a culture grown in collagen for 18 d; *M*, a matched culture to which Matrigel was added on day 10. Gel shifts were used for comparison of transcription factors. Each compar-



ison shows matched concentrations of nuclear extract proteins. Note that Matrigel induces a small decrease in AP1 and HNF4 expression, a small increase in the upper NFκB band, and a marked reduction of C/EBP species. Supershift analysis was used to distinguish between the different species of C/EBP. Protein concentrations were modified to show band shifts of approximately equal strength; the preparations are otherwise the same as in *A*. Antibodies to C/EBPα and C/EBP showed clear supershifting in both preparations and densitometric analysis showed similar proportions of C/EBPα and C/EBPβ in both. Note a lower gel shift band which does not supershift with either antibody. This unidentified C/EBP species is increased in the Matrigel nuclear extract. Bars: (*B*) 5 μm; (*C*) 2 μm.

of the endoplasmic reticulum around mitochondria, bile canaliculi, and presence of glycogen. Preparations of mRNA were made from cultures exposed to Matrigel for 10 d (days 8–18 in culture). The expression of albumin was compared between day zero in culture (immediately after collagenase perfusion), at day 8 in culture (before the overlay by Matrigel), and cultures at day 3 and 7 after Matrigel overlay (Fig. 5 *D*). Addition of Matrigel had dramatic increases in expression of albumin mRNA, compared to control cultures in which it was barely detectable. We also measured the effect of Phenobarbitol (PB) on the levels of cytochrome P450 IIB1 mRNA in the Matrigel-treated cultures (Fig. 5 *E*). Matrigel was added to the cultures at day 8. PB was added 2 d later (day 10 of culture). The cells were harvested 5 d after addition of PB (day 15 in culture). Addition of PB induced cytochrome IIB1 mRNA only in the Matrigel-treated cultures. Induction of this mRNA by phenobarbitol is typical of hepatocytes and does not occur in any other cell (Michalopoulos et al., 1976). Typically most hepatocyte cultures lose the capacity to respond to PB. This finding is additional evidence that addition of Matrigel to the cultures of proliferating hepatocytes induces a mature hepatocyte phenotype, as attested by the electron microscopic structure shown in Fig. 5, *B* and *C*). The expression of cytokeratin 19 (CK19), a bile duct marker (Sirica 1992, 1995) expressed by the proliferating hepatocytes before introducing differentiating conditions (Fig. 3 *B*), also ceased after addition of Matrigel, in contrast to the differentiation toward ductular lineage (see Fig. 6 *E* below) in which CK19 continued to be expressed even in nonproliferating ductular cells. The above changes in gene expression patterns were assessed by detection of the expression of the specific mRNA. The presence of the actual proteins was not directly assessed. The presence of these proteins is reasonable to assume, but not directly proven. Transcription factor changes were also assessed and the results are shown in Fig. 5 *F*. Matrigel induced a marked reduction in C/EBP expression and a smaller decrease in HNF4 and AP1 expression. The reduced C/EBPβ expression represented a reversion from the high level of the cells cultured on collagen to the level observed in normal hepatocytes; however, the expression of C/EBPα was considerably lower than in normal hepatocytes. This is in contrast with other previous studies in which increased differentiation of hepatocytes was associated with increased levels of C/EBPα (see Discussion).

We measured DNA synthesis in the cultures exposed to Matrigel and we found substantial decrease. To assess whether differentiation to mature hepatocyte morphology required DNA synthesis, we added 20 mM hydroxyurea. This has been shown to abolish scheduled semiconservative DNA synthesis in hepatocytes (Michalopoulos et al., 1978) by inhibiting ribonucleotide reductase. Hydroxyurea was added to cultures before the Matrigel overlay and maintained throughout the next 5-d period. DNA synthesis was decreased down to 3.93% of the control (without hydroxyurea) in the cultures maintained in the absence of Matrigel and down to 6.27% of control (without hydroxyurea) in the cultures maintained in the presence of Matrigel. Though DNA synthesis was decreased down to 6.27% of control (+Matrigel, no hydroxyurea) levels, the conversion of the proliferating hepatocytes to mature hepatocyte morphology was entirely unaffected and involved the entire population (data not shown).

HGF/SF (but Not TGFα or EGF) Induces Proliferating Hepatocytes to Differentiate into Ductular/Acinar Structures in Type I Collagen Gels

Hepatocytes maintained between two collagen gel layers retain their morphology and differentiation for prolonged time periods (Michalopoulos et al., 1975; Dunn et al., 1992; Michalopoulos et al., 1993). This is typical of many other epithelial cell types (Hamamoto et al., 1988). We have shown in previous studies that hepatocytes maintained in collagen gel sandwiches in cultures with conventional media containing HGF/SF undergo intense proliferation, form prominent projections, and eventually become organized in structures reminiscent of the hepatic plates (Michalopoulos et al., 1993). We examined in this study the behavior of hepatocytes maintained between two layers of collagen gel as previously described, but in the presence of HGM supplemented with either HGF/SF or EGF. We noticed that in the EGF supplemented media hepatocytes underwent the typical phenotypic transitions as described above. On the other hand, in hepatocytes in HGM supplemented with HGF/SF alone, following expansion of cells identical in appearance to the proliferating hepatocytes described above, there appeared multiple duct-shaped structures between days 10 and 15. These became prominent and encompassed most of the cells present in the cultures. Starting from approximately day 10 and by day 15 most of the cells in the culture were arranged in such ductular structures. The appearance of these structures is shown in Fig. 6 *A*. Histologic sections are shown in Fig. 6 *B* (light microscopy) and Fig. 6, *C* and *D* (electron microscopy). The structures had a ductular or acinar configuration. Some of the cells surrounding these structures were very attenuated and had light and electron microscopic appearance identical to bile duct epithelium. Others however are larger and resemble more the ductular hepatocytes described in previous studies of in vivo models (Gerber et al., 1983). The proliferation of cells (data not shown) under either EGF or HGF/SF in the collagen gel sandwiches was much less (<25% at the highest peak) than that seen in the cultures on plastic coated with dry collagen. Most proliferation ceased by day 10 and the ductlike structures appeared after cell proliferation had ceased (days 10–15). Ductular acinar structures were also noted in these cultures when HGF/SF and EGF were combined but were fewer than with HGF/SF alone. As with the Matrigel overlay, addition of hydroxyurea to inhibit DNA synthesis (inhibition down to 5.1% of control) did not affect the formation of the ductular structures (data not shown). As described from previous studies (Fausto, 1994; Gerber et al., 1983) with ductular hepatocytes in vivo, these cells maintain gene expression patterns characteristic of the ductular hepatocyte phenotype. Fig. 6 *E* demonstrates that these cells express bile duct characteristic cytokeratin 19 (Sirica 1992, 1995). A small amount of albumin expression was also retained, consistent with the presence of hepatocyte-like cells within the ductular structures (Fig. 6 *D*). Please note that the ductular cells maintain CK19 expression in the nonproliferating state in contrast to the cells

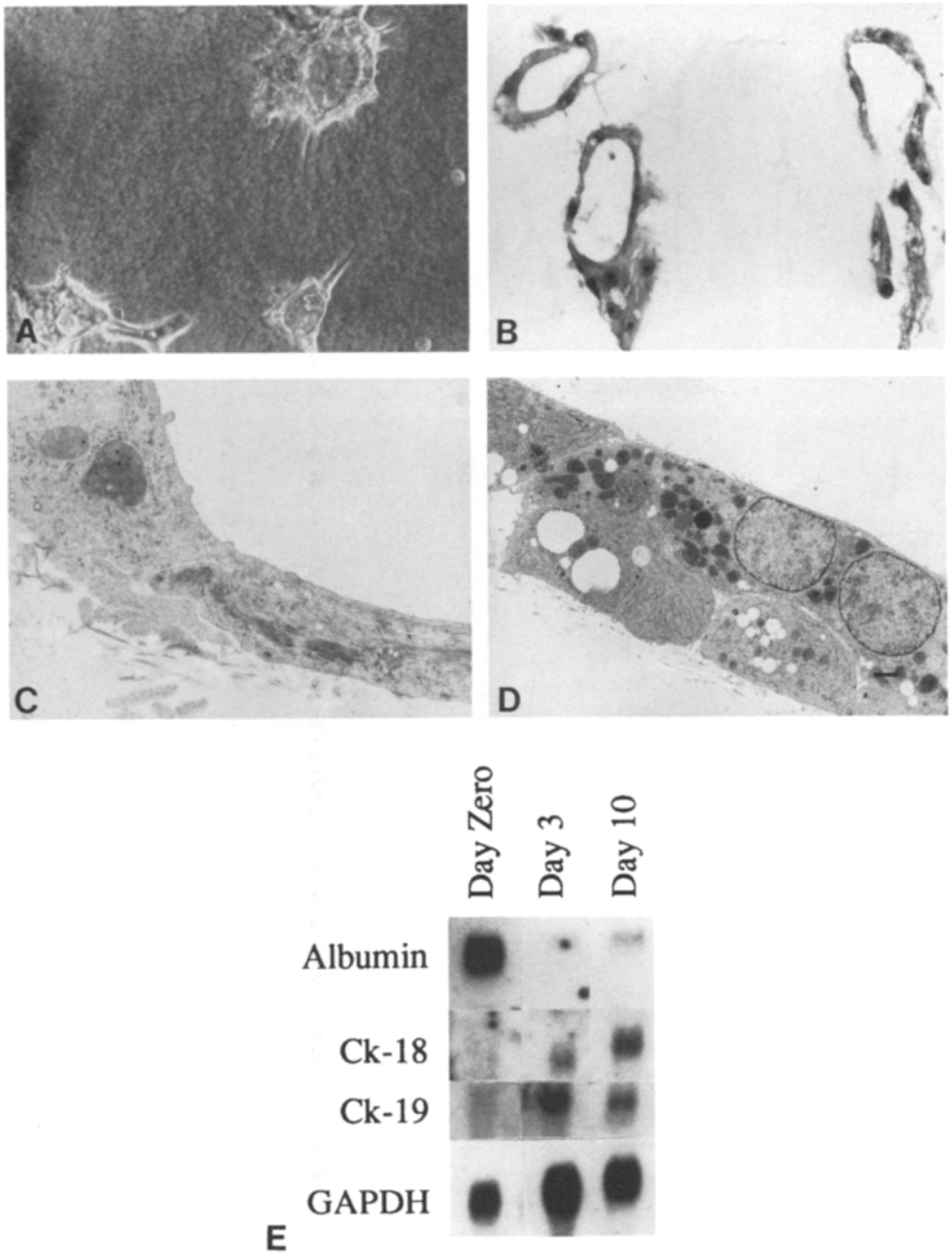


Figure 6. Formation of ductular/acinar structures in cultures kept from the beginning between two type I collagen gel layers in the presence of HGF/SF. Photograph takes from 15-d cultures. (*A*) Phase contrast photomicrograph (100×) showing the appearance of the ductular structures surrounded by collagen fibrils. (*B*) Photomicrographs (100×) of paraffin sections of the above gels stained with hematoxylin and eosin. Note the heterogeneous size of the cells surrounding the ductules. (*C* and *D*) Electron micrographs of cells surrounding the same lumen but in different locations around the lumen. The cells in Fig. 6 *C* have a morphology similar to bile duct epithelium. The cells are attenuated, have long parallel contacts, are joined by many desmosomes and have abundant keratin intermediate filaments. The cells in Fig. 6 *D*, on the other hand, resemble more the hepatocyte phenotype. They have more prominent cytoplasm with rough endoplasmic reticulum and mitochondria, densely stained secondary lysosomes and fewer filaments. (*E*) Expression of cytokeratins 18 and 19 increases in cultures with ductular/acinar structures whereas albumin is only slightly expressed.

differentiating toward the mature hepatocyte lineage which cease expressing this bile duct marker (Fig. 8 *D*).

Association of HGF/SF and c-met Expression in Embryonic Liver with Formation of Acinar/Ductular Structures

The formation of acinar/ductular structures by hepatocytes occurs often in situations associated with hepatic development of neoplasia. In a recent review (Stamatoglou and Hughes, 1994) the formation of acinar/ductular structures during embryonic development was described as a stage preceding the formation of hepatic plates. In view of our findings mentioned above and also in view of recent findings showing impaired hepatic development and embryonic death in mice homozygous for deletions in the HGF/SF gene (Schmidt et al., 1995), we studied expression of HGF/SF and c-met by in situ hybridization in relation to histology of the embryonic liver. Fig. 7 *B* shows the prominent appearance of acinar/ductular structures in embryonic hepatocytes at the time when there is high expression of HGF/SF and c-met (Fig 7, *C* and *D*) during rat liver embryogenesis.

Sustained Growth and Population Expansion of Human Hepatocytes in HGM, in the Presence of HGF/SF and EGF

Though human hepatocyte cultures have not been characterized as extensively as those of the rat, the literature available has shown that these cells also undergo a limited round of DNA synthesis after stimulation by growth fac-

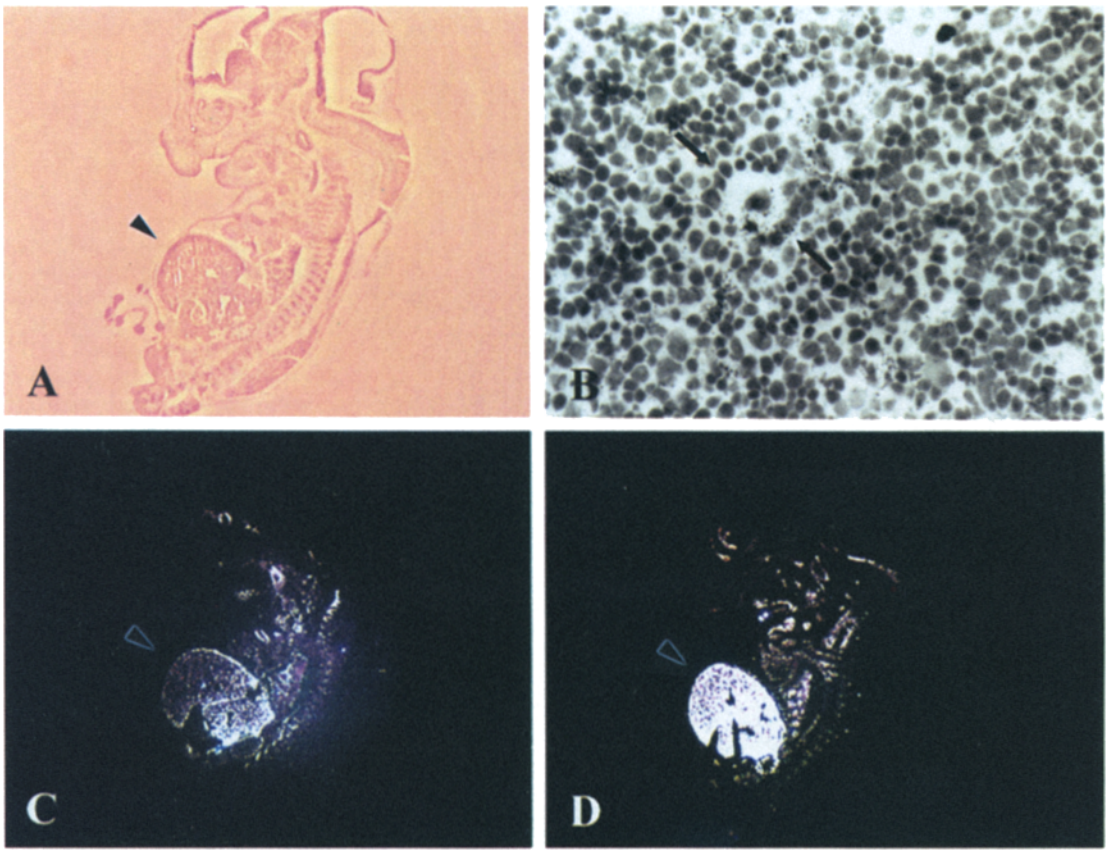


Figure 7. (*A*, *C*, and *D*) Serial sections through a rat embryo at day 16 of gestation. *A* is a frozen section stained by hematoxylin and eosin. *C* and *D* are dark-field photomicrographs of in situ hybridization with anti-sense riboprobes for HGF/SF (*C*) and c-met (*D*). The expression of both HGF/SF and c-met is prominently localized over the embryonic liver (shown with arrows) and also, but less so, over other embryonic structures. In *B*, a photomicrograph was taken from the embryonic liver of *C*. Multiple ductular structures (one shown with two arrows) with characteristic lumens are seen, surrounded by embryonic hepatocytes overlaid by silver grains indicating c-met expression.

tors in culture (Ismail et al., 1991). We tested the response of human hepatocytes to HGF/SF and EGF in HGM medium. Results similar to those described for rat hepatocytes were found in primary cultures of human hepatocytes. Variation was seen between cultures, with best results seen in preparations of higher than 60% viability and donor age younger than 20 years (data not shown).

Discussion

Phenotype of Proliferating Hepatocytes and Lineage Relationships to Hepatocytes and Bile Ducts

The behavior of parenchymal hepatocytes in standard primary culture, including their phenotypic changes and their limited proliferation potential, has been described in many systems (for review see Michalopoulos et al., 1986). The loss of gene expression markers characteristic of mature hepatocytes, the decrease in C/EBPα and the increase in C/EBPβ associated with proliferation, have also been described in earlier systems (Rana et al., 1994). The novel aspect of our findings is that conditions have been defined that allow the progeny of proliferating hepatocytes to clonally expand and increase their population size. The unexpected finding is the rapid reversal to hepatocyte phenotype in the absence of substantial DNA synthesis in the proper matrix and growth factor environment. More unexpected is the capacity of these cells to generate ductular structures, also in the absence of substantial DNA synthesis. The issues to be discussed relate to the conditions that allow clonal growth, the nature of the phenotypic changes observed, the lineage relationship between hepatocytes, proliferating hepatocytes, and bile duct epithelium, the role of HGF/SF as a determinant for formation of ductular structures from proliferating hepatocytes, and the implication of these findings for hepatic embryonic development and recovery from fulminant hepatitis.

Cell Populations in the Primary Cultures

The main cell type in the early cultures is definitely the parenchymal hepatocyte. These cells are derived by standard two step collagenase perfusion of rat and human liver (Seglen, 1976), which yields 98% parenchymal hepatocytes (Seglen, 1976). The electron microscopic and phase contrast pictures shown in Fig. 2 also demonstrate the hepatocyte identity of these cells. Approximately 2% of the cells in a standard collagenase perfusion are not parenchymal hepatocytes. They comprise a mixture of Ito cells, bile duct cells, endothelial cells, and Kupffer cells. From all

that is known about the nature of hepatic cell types, it is highly unlikely that Ito cells, endothelial cells, or Kupffer cells would be the source of the cell types seen in our primary cultures at the end of cell proliferation at culture day 10. The cells comprising our cultures at all times have a very high expression of hepatocyte-associated transcription factors HNF1, HNF3, and HNF4, factors not known to be associated with either of the above three cell types. Bile duct epithelium on the other hand has been shown to express hepatocyte-associated transcription factors during liver regeneration and when hepatocyte proliferation is suppressed by AAF (Nagy et al., 1994). Bile duct epithelium is one of the minor contaminants of the primary cultures estimated to be less than 0.5% of the overall population (Seglen, 1976). In view of the findings that the proliferating hepatocytes (98% of the cells at start point) can give rise to a population massively composed of ductular structures at day 10, we need to address the issue of whether the cells at day 10 are not derived from hepatocytes but from proliferating bile duct epithelium (<0.5% of cells at start point). Careful examination of the quantitative findings of the data presented shows that it is impossible for such a minor contaminant to account for the findings. If only a minor population of cells was proliferating, then there should be a gradual increase of labeling index from <0.5% to a progressively larger percentage by day 10. This is not the case, however, as shown in Fig. 1 *A*. The labeling index is low by day 2 but it rapidly increases to ~80% at day 4 and stays at that level until day 10. The nuclear labeling at day 4 until day 10 is diffuse. From the above considerations, it is mathematically evident that the cells at day 10 are derived from proliferation of the vast majority of the cells present at day 2, i.e., the parenchymal hepatocytes.

Another issue which needs to be addressed is the percentage of true hepatocytes which are capable of producing clones of proliferating cells. After transfection of cells with lac Z, the single cells at day 3 shown in Fig. 2 *F* are typical parenchymal hepatocytes expressing lac-Z. The same type of cells give rise to patches of labeled cells at day 10. The distribution of cells positive for expression of lac-Z at day 3 was entirely random. Since overall the percentage of lac-Z–expressing cells (~20%) did not change from day 3 to day 10, it is clear that the progeny of the lac-Z–expressing cells arranged in clusters at day 10 is derived from growth of the 20% of the cells labeled at day 3. Since 98% of the cells at day 3 are parenchymal hepatocytes, most of the lac-Z–expressing cells at day 10 must be derived from parenchymal hepatocytes. The findings with lac-Z–infected cells also corroborate the conclusions we have reached by direct microscopic observation of the cell populations in the cultures. The morphologic changes associated with cell proliferation are gradual and they affect the entire cell population. These changes have been described in the past for other primary cultures (Chapman et al., 1973) and they primarily appear as gradual loss of granularity from the hepatocyte cytoplasm by phase contrast. There is no evidence of selective cell loss of specific hepatocyte populations.

As stated in the Results, the proliferating hepatocytes in the proper matrix and growth factor environment generate mature hepatocytes or ductular structures even when DNA synthesis is completely abolished by addition of hydroxyurea. The vast majority of the cells present as proliferating hepatocytes converts to either the ductular structures or the mature hepatocytes under these conditions. This demonstrates that the proliferating hepatocytes, derived from the mature hepatocytes of the original inoculum, can become mature hepatocytes or form ductular structures. The molecular markers used demonstrate that the differentiated hepatocytes express albumin, respond to phenobarbitol by induction of cytochrome P450 and cease expressing CK19, an in vivo marker for mobile duct cells (Sirica 1992, 1995).

The finding that hepatocytes can give rise to two types of progeny suggests that at least under culture conditions, some if not most of the hepatocytes can function as bipotential stem cells. Such cells are expected to give rise to hepatocytes and ductular type cells. The term "hepatoblast" (etymologically defined as cells that can give rise to liver) has been used to describe the embryonic cells that differentiate both toward hepatocytes and bile duct epithelium (Fausto et al., 1993). In adult rats such a term is not frequently applied. The closest cell type with a bipotential differentiation are the oval cells or their precursors. These are cells that emerge when liver is stimulated to regenerate but the proliferation of the normal hepatocytes is suppressed (Evarts et al., 1989). Oval cells proliferate diffusely. They are often arranged in a ductular configuration and eventually differentiate to mature small hepatocytes when the inhibitory stimulus is removed (Evarts et al., 1989). The findings of our study show that regardless of other cell types, mature hepatocytes can also give rise to cells that can behave as hepatoblasts.

Transcription Factor Changes Associated with Proliferating Hepatocytes and Their Redifferentiation to Mature Hepatocytes

Consideration of the changes in transcription factors also supports the comparison between our proliferating hepatocytes and hepatoblasts. The proliferating hepatocytes express high levels of most transcription factors associated with the mature hepatocyte phenotype (Tronche, 1992). Specifically C/EBPβ, HNF1, and HNF3 remained normally expressed and HNF4 increased. HNF3β and HNF3α have been described as the earliest expressed liver transcription factors during embryonic development (Ang et al., 1993). C/EBPα levels are low during the early stages of embryonic development, when the other transcription factors are expressed (Nagy et al., 1994). C/EBPα decreases dramatically after stimulation of liver regeneration (Flodby et al., 1993) or entry of hepatocytes into proliferation in primary culture (Mischoulon et al., 1992). Characteristic patterns of induction of the C/EBP family members in the sequence of δ, β, α have also been described in postnatal liver development (Diehl et al., 1994; Diehl and Yang, 1994). It is unclear, however, whether the absence of C/EBPα is the sole factor that significantly affects the phenotype of the cultured cells, since they still express abundant C/EBPβ. In most cases, C/EBPα and C/EBPβ show almost identical binding and activation properties, although Lee et al. (1994) have described a cooperative interaction with transcription factor Sp1 that occurs with C/EBPβ but not C/EBPα. This

might suggest selected, perhaps subtle, alterations in phenotype associated with the differential expression of C/EBPα and C/EBPβ, but not a drastic alteration in phenotype associated with loss of C/EBPα expression. Loss of the differentiated phenotype might be more directly associated with high AP1 expression, which has been shown to directly suppress activity of the albumin gene enhancer (Hu and Isom, 1994). The increase in AP1 and NFκB is characteristic of proliferating cells. Their levels in liver embryogenesis or oval cell development have not been directly addressed, although it has been shown that liver development fails in mice homozygous for a deletion in *c-fos* (Hilberg et al., 1993). The elevated HNF4 expression is also of interest, because it is mostly due to a second band shift, slightly higher and distinct from the usual HNF4 band. Other liver-enriched factors are expressed as multiple distinct family members, but HNF4 has so far been described only as a single factor (Sladek et al., 1990; Xanthopoulos et al., 1991). The upper band could represent a transcriptional or posttranscriptional modification of HNF4, a new factor that binds the HNF4 site, or a heterodimer of HNF4 with another factor. The doubleband HNF4 gel shift pattern is unique to these cultures.

As mentioned in the results, several previous studies have associated increased hepatocyte differentiation in culture with increase in levels of C/EBPα (Mischoulon et al., 1992; Rana et al., 1994). In these studies addition of Matrigel resulted in enhanced hepatocyte differentiation, increase in levels of C/EBPα and decrease in levels of AP1. In our study, there was no increase in C/EBPα when Matrigel induced differentiation of proliferating hepatocytes into mature cells. The reasons for this discrepancy are not clear. On the other hand, the decrease in AP1 and NFκB levels is compatible with previous literature, in which both of these factors are associated with enhanced hepatocyte proliferation (Tewari et al., 1992; Rana et al., 1994).

Media Components Important for Clonal Growth

The components of the medium that allow these cells to proliferate beyond the limitations of the standard media are currently under further study. A cell culture medium similar in many respects to ours was previously presented by Mitaka et al. (1992*a*,*b*, 1993*a*,*b*). This medium also contained nicotinamide but it allowed formation of only small colonies of cells which coexisted with mature hepatocytes in dense cultures. These colonies did not form when sparse cultures of hepatocytes were used and expansion of the population comparable to the results presented in our paper was not demonstrated. The composition of HGM differs from the medium described by Mitaka et al. (1991, 1992*a*,*b*, 1993*a*,*b*) in many significant ways. The most important difference may be the presence in HGM of saturated diferric transferrin. As shown in Table I *B*, when unsaturated transferrin is used instead of the saturated diferric type, the growth of the cells was significantly inhibited. Addition of iron did not substitute for the effect of diferric transferrin. Another important difference is the addition of glucose in HGM. Galactose alone, used in the medium by Mitaka et al. (1991, 1992*a*,*b*, 1993*a*,*b*) is not sufficient for full proliferative effects. We also added arginine in addition to ornithine. Arginine in past work was removed from hepatocyte media in order to prevent contamination of cultures by nonparenchymal cells (Leffert et al., 1977). As the proliferating hepatocytes lose specific hepatocyte related functions, it is likely that the pathways generating arginine through urea cycle would diminish their activity or disappear altogether. Thus the absence of arginine might also be rate limiting for the growth of the proliferating hepatocytes. Differences in the detail composition of trace elements etc. cannot be deduced from the existing publications but they also may be critical. We also found, as did Mitaka et al. (1991, 1992*a*,*b*, 1993*a*,*b*) that DMEM supports growth of hepatocytes more than the standard MEM (data not shown).

Role of Diferric Transferrin and Nicotinamide

The role of diferric transferrin and nicotinamide in maintaining hepatocyte growth and function is well documented. Nicotinamide was shown in the past to promote maintenance of cytochrome P450 types in hepatocyte cultures (Paine et al., 1979), inhibit the expression of some fetal markers such as GGT (Rosenberg et al., 1982) and prolong hepatocyte function maintenance in primary culture (Inoue et al., 1989). It was also shown to have effects on hepatocyte DNA repair (Althaus et al., 1980) as well as to prolong hepatocyte proliferation in standard media (Mitaka et al., 1991). The mechanism mediating these effects is not clear. Nicotinamide appears to not be used as a source of replenishment of NAD and NADP in isolated hepatocytes (Bender and Olufunwa, 1988). A potential interaction between NAD and diferric transferrin appears to occur at the hepatocyte plasma membrane (Morre et al., 1991). These studies have shown that most of the iron (70%) in hepatocytes exist in an extracellular pool of diferric transferrin bound to its plasma membrane receptor. It participates in mediating plasma membrane redox activities with a nicotinamide adenine dinucleotide (NADH) reductase and together the two are crucial in maintaining the functions of the Na^+/H^+ antiport, a channel involved at the early steps of stimulation of DNA synthesis by growth factors.

Role of the Matrix in Proliferating Hepatocyte Growth and Differentiation

Whereas no major differences in growth rate based on matrix coating of the plates were seen, the matrix environment seems to have a more defining role on the differentiation of the cells once growth ceased. Addition of Matrigel promoted a return of the differentiation to the mature hepatocyte phenotype. This is in agreement with previously described effects of Matrigel causing induction of polarity and canalicular differentiation in nonproliferating hepatocyte cultures (Musat et al., 1993). The "spontaneous" return to mature hepatocytes seen in old cultures with focal fibroblast overgrowth is also probably in part matrix dependent. As mentioned above, the "fibroblasts" seen in hepatocyte cultures actually derive from Ito cells of the liver (Maher et al., 1988), the cells responsible for most of the matrix production. It was recently shown that Ito cells express mRNA for collagen types III and IV as well as for laminin, whereas collagen type III, type I, and fibronectin mRNA were expressed by parenchymal hepa-

tocytes (Geerts et al., 1993). Ito cells that have transformed to fibroblast-like morphology also produce large amounts of collagen type I, fibronectin, and laminin. It is highly likely that the focally growing fibroblasts produce liver related matrix that directs the differentiation of the immature proliferating hepatocytes back to the mature parenchymal phenotype. The effect of Matrigel may be due to its high laminin content (Kleinman et al., 1982). Other components of Matrigel, however, may be responsible for this effect. Further studies with matrix components will be performed to study the effects of specific matrix proteins. It should be noted that Ito cells synthesizing matrix play a crucial role in the differentiation of the oval cells back to mature hepatocytes. Ito cells grow together with the oval cells and provide the background on which the differentiation to hepatocytes occurs through production of matrix and growth factors (Evarts et al., 1993).

HGF/SF and Its Receptor as the Key Molecules Controlling Formation of Acinar/Ductular Structures from Hepatocytes: Implications for Fulminant Hepatitis and Embryonic Development

Acinar/ductular structures composed of cells with properties intermediate between hepatocytes and bile ducts are very commonly seen during different aspects of growth of embryonic, adult normal, and neoplastic liver. Stamatoglou and Hughes (1994) have documented that these structures are intermediate stages between apolar immature hepatocytes and mature hepatocytes arranged in hepatic plates. Of the various growth factors used in our study the only one that induced formation of these structures was HGF/SF. When EGF was added with HGF/SF the frequency of acinar/ductular structures substantially diminished. The association of HGF/SF with formation of ductular structures has been documented in several recent studies. The association of expression of the HGF/SF receptor (MET) with formation of ductular structures was also recently shown in kidney derived cell lines (Montesano et al., 1991), in the mammary gland (Tsarfaty, 1992; Soriano et al., 1995) and in nonparenchymal liver derived cell lines (Johnson et al., 1993). EGF or TGFα alone did not induce these structures in the hepatocyte cultures. These findings have relevance to the histology of fulminant hepatic failure. Structures composed of ductular cells with hepatocytic markers are also seen at the end stages of fulminant hepatitis. When most of the hepatocytes die, the only proliferating cells left are the "ductular hepatocytes" arranged in ductular structures near residual portal triads. Independent studies have shown that during the same stage of fulminant hepatitis when ductular hepatocytes appear, plasma HGF/SF levels are extremely high (Tsubouchi et al., 1989). Our findings suggest that the acinar/ductular structures seen in fulminant hepatitis are induced by the extremely high levels of HGF/SF in the plasma. Since previous studies (Evarts et al., 1989; Vandersteenhoven et al., 1990) have shown that these cells can become hepatocytes, it is conceivable that the very high levels of HGF/SF in the plasma block further differentiation of these cells into mature hepatocytes, thus inhibiting liver regeneration and causing the irreversible hepatic failure associated with the high mortality of this syndrome.

In a recent publication it was shown that lack of HGF/SF is critical for embryonic development and that one of the key organs whose growth was drastically inhibited was liver (Schmidt et al., 1995). Homozygous null mice for HGF/SF died at day 16.5 of gestation. The photographs of Fig. 7 showing the strong expression of HGF and c-met mRNA in embryonic liver were taken from rat embryos at day 18. The studies by Stamatoglou and Hughes (1994) show that appearance of ductular/acinar structures in the embryonic hepatocytes at this stage is required for the formation of the mature hepatocyte phenotype. Our findings suggest that the reason for inhibition of hepatic embryogenesis in the HGF/SF-null homozygous mice is the inability, in the absence of HGF/SF, to undergo the phenotypic transitions from the diffuse unstructured parenchyma of apolar embryonic hepatocytes to acinar ductular structures, a transitional stage before the formation of mature hepatic tissue.

Hepatocytes as the Cell of Origin for Hepatocytic Lineages

The findings of this paper raise questions on the origin of multipotential cells often seen in the liver. These cells are seen in numerous conditions, both in experimental animal models as well as in human disease. They have been called oval cells in rodent carcinogenesis and ductular hepatocytes in fulminant hepatitis in humans (Evarts et al., 1989; Gerber et al., 1983). The tendency of these cells to aggregate and form ductular structures has made most of the workers in the field believe that the bile ductular or periductular cells are the origin of these populations. The findings in this paper clearly demonstrate that mature hepatocytes can also give rise to such cells. We have shown that the immature cells derived from hepatocytes can multiply and expand in a clonal fashion. Additionally, these cells have markers of gene expression that relate to both hepatocytes and bile ducts. We also show that these cells can enter back into differentiation pathways that can lead back to hepatocytes or to formation of ductular structures. Matrix components such as laminin and type I collagen and growth factors such as HGF/SF and EGF and TGFα appear to participate in the control of these transitions. The transcription factor changes associated with this transition from mature hepatocytes to proliferating hepatocytes with apparent bipotential growth are rather conservative. Loss of C/EBPα and increase in NFκB and AP1 are the major changes observed. The other hepatocyte specific factors (HNF1, HNF3, HNF4) continue to be present. The presence of hepatocyte specific transcription factors at the same time as the cells express bile duct specific (CK19) and rat liver stem cell specific (CK14) (Bisgaard et al., 1994) cytokeratins also underscores the bipotential nature of these cells. The expression of α-fetoprotein is also associated with the multipotential stem cells (Sell, 1994). The emergence of acinar/ductular structures from hepatocytes clearly suggests that hepatocytes, at least in part, can account for the origin of the multipotential cells seen in liver. This is consistent with previous findings with transgenic animals in which hepatocytes have been shown to have almost unlimited clonal potential (Rhim et al., 1994), though the transcription factor profile of these cells participating in the formation of hepatocytic clones in vivo has not been described. It is not clear from our findings, whether hepa-

tocytes in all three acinar zones have the capacity of generating such cells. Most of the studies suggest that periportal hepatocytes are more likely to enter into proliferation. Ductular/acinar structures are seen more commonly in the first acinar zone (periportal). Detailed dissection of the periportal and pericentral hepatocytes by established techniques needs to be pursued to further study this issue. The standard collagenase perfusion used in these studies recovers >90% of all the hepatocytes present in the liver and does not allow for this distinction to be made. Whether all hepatocytes can give rise to such cells with bipotential differentiation remains unclear. The data from Fig. 1 *A* and 2, *F* and *G* suggest that the majority of hepatocytes can give rise to cells with bipotential differentiation. Our findings cast hepatocytes and HGF/SF in a new role in which cellular phenotypic transitions governed by matrix and maintained by morphogenetic effects induced by high levels of HGF/SF provide new pathways for liver injury repair and embryonic development. The system described in this study allows for the first time for clonal expansion of rat and human hepatocytes, and it should be of relevance to studies using hepatocytes as vehicles for gene therapy or as cells for the build up of artificial liver devices.

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