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Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor α

(liver/hepatocarcinogenesis/growth factors/liver regeneration/cell culture)

JUSTINA C. WU*, GLENN MERLINO†, AND NELSON FAUSTO*‡

*Department of Pathology and Laboratory Medicine, Box G, Brown University, Providence, RI 02912; and †Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Hepatocytes are extensively used in studies of gene regulation but cannot be maintained in long-term culture as replicating, differentiated cells while remaining nontumorigenic. We have derived two hepatocyte lines from livers of transgenic mice overexpressing transforming growth factor α, a potent hepatocyte mitogen, which overcome these limitations. The transgenic hepatocytes were maintained for ≥2 months in serum-supplemented primary culture and gave rise to cell lines, of which two (AML12 and AML14) have been cultured for >1.5 years (>80 passages). Both lines have typical hepatocyte features such as peroxisomes and bile canalicular-like structures, do not grow in soft agar, and are nontumorigenic in nude mice. Like normal hepatocytes, AML cells express high levels of mRNA for serum (albumin, α_1-antitrypsin, and transferrin) and gap junction (connexins 26 and 32) proteins, secrete albumin, and contain solely isozyme 5 of lactate dehydrogenase. After extensive passaging, AML12 cells continue to strongly coexpress hepatocyte connexin mRNAs but do not display nonparenchymal cell markers. Although mRNA levels for some serum proteins progressively fall, high expression in late AML12 cultures may be regained by passage in serum-free medium. The AML14 line loses expression of both differentiated markers and transgene mRNA with extended passaging, and hepatocytic traits are only partially restored by passage in serum-free medium. These differentiated, nontumorigenic cell lines should serve as models in which to study hepatocyte growth and differentiation.**

Mammalian hepatocytes have long been used to investigate mechanisms of cell growth, differentiation, and tumorigenesis and as vectors for gene therapy. These studies have been hampered because dissociated hepatocytes rapidly lose their cell-specific functions, are short-lived, and have a limited ability to replicate in culture. Longer-term differentiated cultures can be maintained with specialized media and substrata or by coculture with nonparenchymal cells, but under these conditions the cells remain nonproliferative (1–3).

Hepatocyte lines which express some tissue-specific markers have been propagated *in vitro* from hepatocellular tumors. More recently, well-differentiated lines have been derived by introducing viral oncogenes into hepatocytes. While useful, these lines have the drawback of either being fully transformed or undergoing transformation with repeated passaging (3–6).

Transforming growth factor α (TGFα) is a polypeptide that regulates normal growth in epithelial tissues, and its overproduction in cells possessing epidermal growth factor receptors (EGFRs) is often correlated with malignant transformation (7–10). TGFα is expressed in the liver during embryogenesis and transiently increases during liver regeneration after partial hepatectomy. Proliferating hepatocytes produce and respond to TGFα in an autocrine manner (11, 12). Constitutive TGFα expression causes liver hypertrophy and increased hepatocyte proliferation in young transgenic mice, followed by the development of multifocal hepatocellular tumors, a process accelerated by c-*myc* overexpression or coexpression of the simian virus 40 large tumor (T) antigen (13–17).

Here we report the establishment of two hepatocyte lines from livers of TGFα-transgenic mice, which have been in culture for >1.5 years (>80 passages), are nontumorigenic, and possess a large complement of differentiated traits. In one line, expression of TGFα and liver-specific genes has been maintained throughout culture and can be upregulated by passaging the cells in serum-free medium.

MATERIALS AND METHODS

Hepatocyte Isolation. Hepatocytes were isolated from livers of 5-month-old male homozygous TGFα-transgenic mice (CD1 strain, line MT42), which carry a human TGFα (hTGFα) cDNA driven by the zinc-inducible metallothionein 1 promoter (13). The liver was perfused by Seglen's method (18) via the inferior vena cava with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, followed by 100 units of collagenase. Hepatocytes (≥90% viable) were purified by isodensity Percoll centrifugation (19).

Cell Culture. Hepatocytes were plated (8×10^6 cells per 100-mm dish) on plastic dishes in Dulbecco's modified Eagle's medium/Ham's F-12 (GIBCO) supplemented with 10% fetal bovine serum (FBS), a mixture of insulin, transferrin, and selenium (ITS; Collaborative Research), 0.1 μM dexamethasone, and gentamicin at 50 μg/ml. After a 2-hr attachment period, the culture medium was replaced and changed every 3–4 days. After 2–3 months, colonies were individually isolated, trypsinized, and expanded; the first subcultures are designated as passage 1 (p1) cells. Cultures were passaged at varying intervals through p6 (total of 4 months in culture) and then regularly every 5–7 days thereafter, or when ≈70% confluent.

Electron Microscopy (EM). For routine EM, cells were processed by standard methods. To demonstrate catalase activity, fixed cultures were incubated in 5 μM diaminobenzidine/0.1% H_2O_2 in phosphate buffer, postfixed in 1% osmium tetroxide/0.15 M sodium cacodylate, and then processed for EM (20).

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Abbreviations: A1AT, α_1-antitrypsin; AFP, α-fetoprotein; Cx, connexin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; LDH, lactate dehydrogenase; p*n*, passage *n*; TGFα, transforming growth factor α; hTGFα, human TGFα; mTGFα, mouse TGFα.

‡To whom reprint requests should be addressed.

RNA Extraction and Northern Blot Analysis. Northern blot analysis was performed on total RNA extracted from cultured cells at 70% confluence (12, 21). Filters were hybridized at 50°C [for the connexin (Cx) 32 probe] or 42°C (all other probes) with the following ^{32}P-labeled probes: a 240-bp *Pst* I cDNA fragment recognizing human and mouse TGFα mRNA; a 2.4-kb human EGFR cDNA insert from pE7 (22); the mouse albumin cDNA insert from pmalb2 (23); a 0.5-kb *Bgl* I–*Bam*HI α-fetoprotein (AFP) cDNA fragment from pBAF700 (24); the mouse α_1-antitrypsin (A1AT) insert in pLivS3 and transferrin insert in pLivS6 (from H. Isom, Pennsylvania State University College of Medicine) (25); the rat Cx 26 and 32 cDNAs in pSP42T and pGEM-3, respectively (from D. Paul, Harvard Medical School) (6, 26); the rat Cx 43 insert in pBS (from E. Beyer, Washington University School of Medicine) (27); and a 1.2-kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA from pRLC-GAP (from J. Tso, X.-H. Sun, and R. Wu, Cornell University). For multiple probings, filters were stripped in 1% (vol/vol) glycerol at 80°C for 3 min before rehybridization.

Immunological and Biochemical Assays. Cells on glass slides were fixed and stained by the avidin–biotion complex (ABC) immunoperoxidase method (Vector Laboratories) using a 1:1000 dilution of rabbit anti-mouse albumin IgG (Cappel Laboratories). NIH 3T3 cells and specimens incubated without primary antibody were negative controls. Albumin immuno-overlays were performed on cells seeded at limiting dilution using 1:64 IgG (4, 28). For analysis of lactate dehydrogenase (LDH) isozymes, 50-μg samples of cell protein were subjected to nondenaturing PAGE and enzyme activity was detected as described (29, 30). γ-Glutamyltransferase histochemistry was performed by the method of Rutenberg *et al.* (31).

Oncogenicity Assays. For soft-agar assays, 10^5 cells in 0.3% agar/growth medium were overlaid onto 0.6% agar in 35-mm dishes; stained, macroscopically visible colonies (≥100 μm in diameter) were scored after 14 days. Spontaneously transformed LE2 (liver epithelial) oval-cell lines served as positive controls (30). Tumorigenicity was determined by injecting 10^7 cells subcutaneously into flanks of 4- to 5-week-old male nude mice. For each cell line, 10 sites were injected and animals were examined weekly for tumors for ≥5 months.

RESULTS

Establishment of TGFα-Transgenic Hepatocyte Lines. Normal mouse hepatocytes cultured in defined serum-free medium do not proliferate but will undergo DNA synthesis in response to EGF, TGFα, or hepatocyte growth factor (1, 32). In contrast, hepatocytes from TGFα-transgenic mice replicate autonomously in primary culture, without exogenous mitogens. However, both normal and transgenic hepatocytes senesce after 7 days in serum-free culture, even when growth factors are added (J.C.W. and N.F., unpublished work).

To overcome these difficulties, we established primary cultures of TGFα-transgenic hepatocytes in medium supplemented with 10% FBS. Under these conditions, the hepatocytes formed semiconfluent monolayers which developed into cord-like patterns. Over the next 3 weeks, as some hepatocytes began to degenerate, 30–60 small colonies per dish, varying in morphology and growth pattern (Fig. 1 *A–C*), arose and grew slowly. After 2–3 months, colonies were individually trypsinized and subcultured to develop cell lines.

Among seven lines which survived to or beyond p5 (Fig. 1*D*), hTGFα transgene mRNA expression was highest in lines 12 and 14, which also expressed albumin mRNA to the greatest degree (>50% relative to cultured hepatocytes). These lines, designated AML12 and AML14, originated from colonies similar to those shown in Fig. 1 *A* and *C*, respectively.

Morphology of AML Lines. After p8–9, the AML12 and AML14 lines retained their epithelial morphologies and grew in cords and patches. AML12 cells were regular, flat, polygonal cells with round nuclei and granular cytoplasm (Fig. 2*A*). As observed by EM, AML12 cells displayed distinctive features of hepatocytes (Fig. 2*C*) such as regular nuclei, abundant mitochondria and rough endoplasmic reticulum, and glycogen deposits. Adjacent cells formed bile canalicular-like structures complete with microvilli and tight junctions, and most cells contained numerous peroxisomes as demonstrated by catalase staining. In comparison, the AML14 line was more heterogeneous (Fig. 2*B*) but contained cells with the hepatocyte ultrastructural features described above (Fig. 2*D*).

Growth Properties and Lack of Oncogenicity of AML Lines. At 6 months in serum-supplemented culture (p13–14), the AML12 and AML14 hepatocyte lines proliferated with doubling times of 37 and 32 hr, respectively. Both lines have been cultivated for >18 months, or >p80, without crisis and are thus immortalized. AML12 cells at very high passage (p69–72, 16 months old) did not display anchorage-independent growth or produce tumors in nude mice, even after undergoing 10 passages in serum-free medium. AML14 cells grew very weakly (0.005% cloning efficiency) in soft agar and were

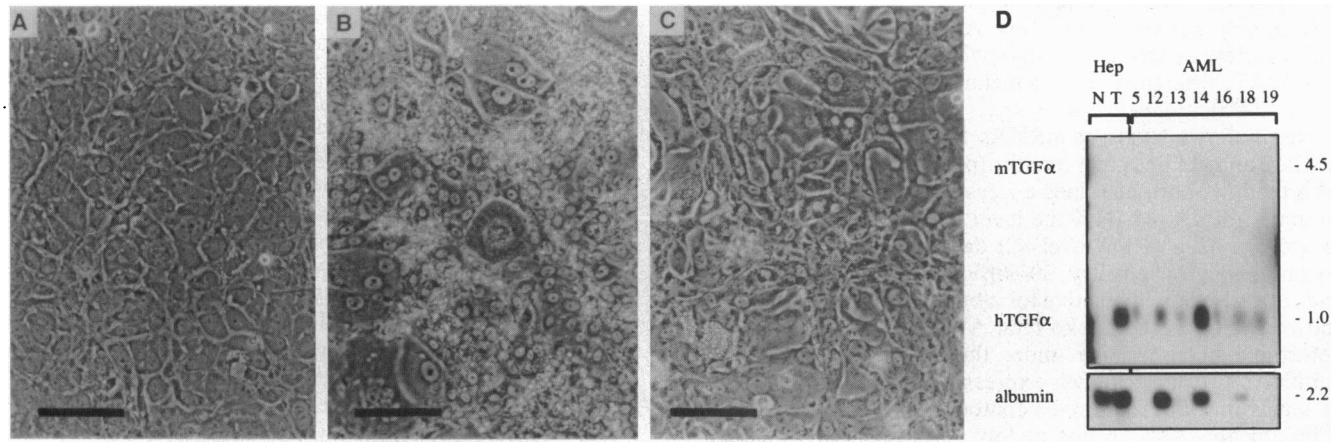

FIG. 1. Colonies arising in primary cultures of TGFα-transgenic mouse hepatocytes maintained for 2 months in medium containing 10% FBS. (*A–C*) Phase-contrast micrographs show representative colony types: flat polygonal cells (*A*), small cells interspersed with giant multinucleate cells (*B*), and mixed colony (*C*). (Bar = 100 μm.) (*D*) Northern blot analysis of 20 μg of total RNA isolated from 24-hr primary hepatocyte cultures (Hep) or TGFα-transgenic mouse liver cell lines at p5–6, which corresponds to 3–6 months in culture (AML). Lanes; N, normal hepatocytes; T, transgenic hepatocytes; AML, seven TGFα-transgenic liver cell lines. Sizes of detected transcripts are indicated. mTGFα, mouse TGFα.

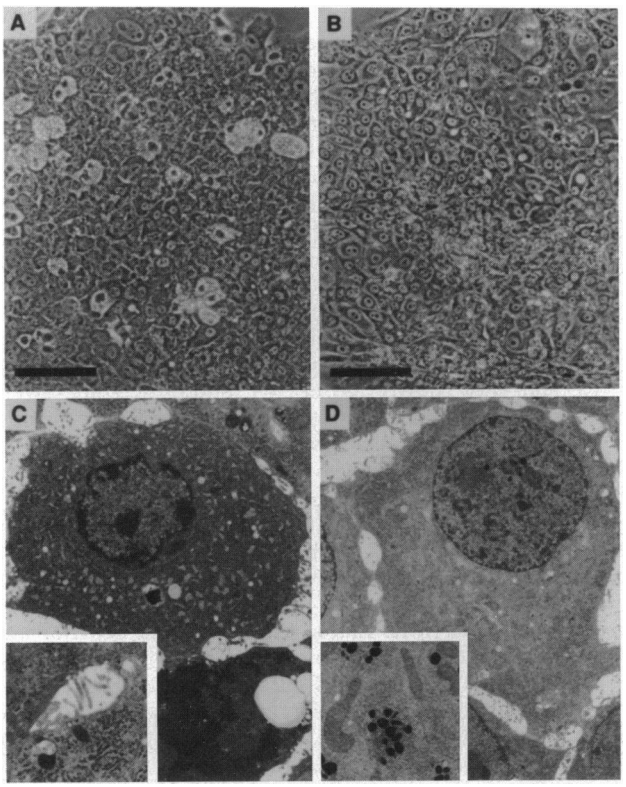

FIG. 2. Morphology of AML12 and AML14 lines. (*A* and *B*) Light micrographs of AML12 (*A*) and AML14 (*B*) cell lines at p8–9. (Bar = 100 μm.) (*C* and *D*) EM views of AML12 (*C*, ×7500) and AML14 cells (*D*, ×3500) at p16–18. Glycogen deposits and vacuoles are apparent in AML12 cells. Both cell lines contain regular nuclei, mitochondria, and abundant rough endoplasmic reticulum, as well as bile canalicular structures (*C Inset*, ×14,300) and peroxisomes (*D Inset*, ×8200).

also nontumorigenic at very late passages. Hence, neither line has transformed even after long-term culture.

Phenotype of AML Lines. The presence of specialized morphological features such as peroxisomes indicated that the AML12 and -14 lines were derived from liver parenchymal cells and had retained differentiated traits. To determine whether the lines also retained hepatocyte functions in these culture conditions, we looked for expression of genes whose products are cell/tissue-specific and are considered hallmarks of the hepatocyte phenotype.

Hepatocyte secretory proteins. Adult hepatocytes synthesize a variety of serum proteins such as albumin, transferrin, and A1AT, whereas AFP is a major product of fetal hepatocytes (3, 25).

Fig. 3 shows levels of mRNAs for these proteins in the AML12 and -14 lines at 4 months (p5–6) in culture and later. At 4 months, both cell lines expressed high levels of 2.2-kb albumin mRNA (34–41% the level of freshly isolated hepatocytes, or 52–63% the level of 1-day cultured hepatocytes). By immunocytochemistry, 30–40% of early-passage AML12 and -14 cells stained positive for albumin (Fig. 4 *A* and *B*). As shown in Fig. 3, the 2.2-kb fetal AFP transcript was barely detectable in either line under the usual conditions. The 1.5-kb A1AT transcript was expressed by AML12 cells (34% of levels in 1-day cultured hepatocytes) and more weakly (17%) by the AML14 line at low passages. Early-passage AML cells also produced 2.2-kb transferrin mRNA at levels exceeding that of primary hepatocyte cultures (113–159%). Thus, even several months after isolation, both AML lines substantially expressed mRNAs encoding adult hepatocyte-specific proteins.

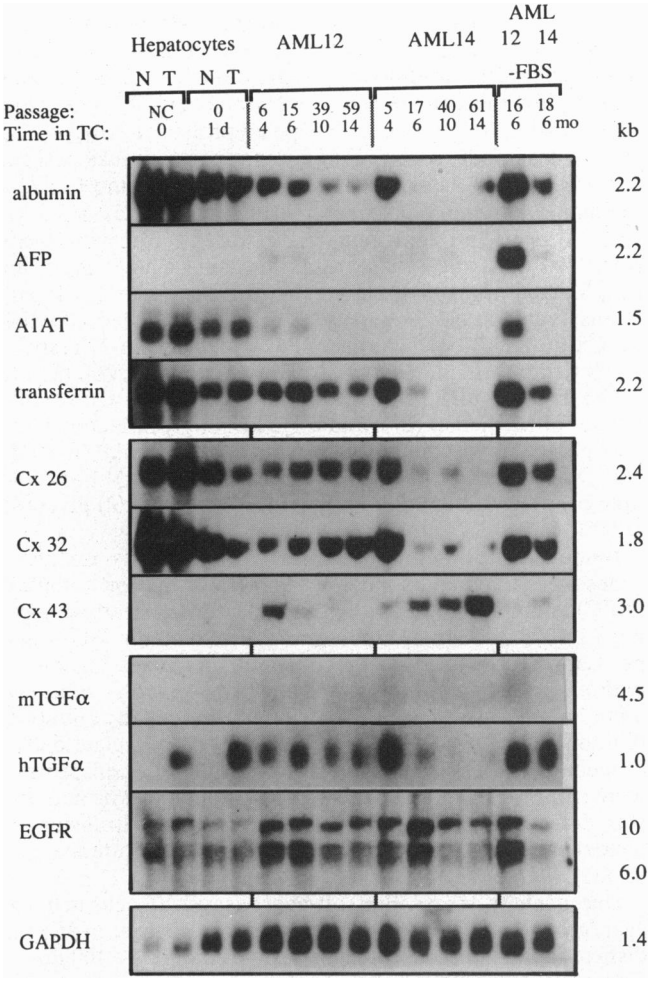

FIG. 3. Northern blot analysis of hepatocyte-specific markers in AML12 and AML14 lines. Samples (20 μg) of total RNA from normal (N) or transgenic (T) mouse hepatocytes [NC, not cultured (freshly isolated); (1 d, cultured for 24 hr)] and AML lines {at the indicated passages and corresponding times after isolation from liver [time in tissue culture (TC)]} were subjected to Northern analysis with the appropriate ^{32}P-labeled cDNA probes. Sizes of detected transcripts are indicated. Most cells were grown under usual culture conditions, in medium with 10% FBS. The last two lanes (−FBS) show RNA from AML12 and AML14 cells passaged twice in serum-free medium. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Hepatocyte connexins. The connexins are a family of gap-junction proteins which are differentially expressed in tissue-specific patterns. Cx 26 and Cx 32 are the only types known to exist in hepatocytes, whereas Cx 43 is found in bile ductules and dedifferentiated liver cell lines (6, 26, 27, 33). At 4 months in culture, both AML lines synthesized the 2.4-kb Cx 26 and 1.8-kb Cx 32 transcripts at levels comparable to that of 1-day cultured hepatocytes (Fig. 3). In AML12, the 3.0-kb Cx 43 message was detectable only at early passages; by 14 months Cx 43 mRNA was absent while both hepatocytic Cx mRNAs remained. By contrast, in the AML14 line, Cx 26 and 32 mRNAs declined with passage as Cx 43 mRNA increased. These results suggest that AML12 retained its differentiated phenotype, whereas AML14 became less differentiated with passage.

Hepatocyte-associated enzymes. LDH is an intracellular enzyme with five distinct isoforms. Hepatocytes produce only the predominant liver form LDH 5, whereas isozymes 4 and 3 are made by nonparenchymal liver cells and oval cells early in hepatocarcinogenesis. γ-Glutamyltransferase is a membrane-bound enzyme expressed in fetal hepatocytes,

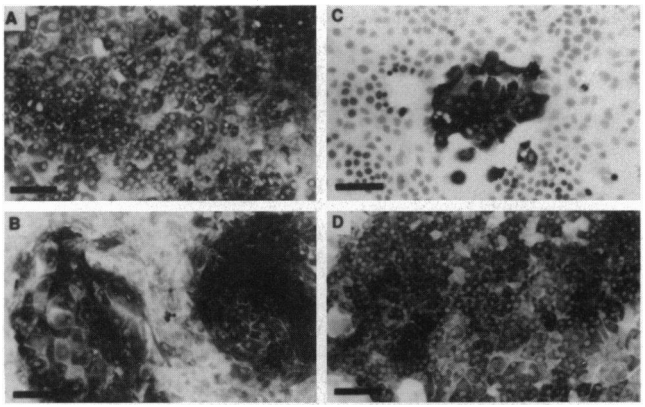

FIG. 4. Albumin immunocytochemistry in AML12 and AML14 lines. AML12 (*A*) and AML14 (*B*) cells at p11 (5 months) were grown in medium containing 10% FBS. AML12 cells at p51–81 (13–18 months) were either cultured in medium with 10% FBS (*C*) or passaged once in serum-free medium (*D*). The presence of albumin is indicated by dark precipitate in the cytoplasm. (Bar = 100 μm.)

bile ductules, and many hepatic neoplasms (29). AML12 and -14 produced exclusively LDH 5, the other isozymes being undetectable in either line. γ-Glutamyltransferase was absent in AML12 cells grown under usual conditions, although ≤10% of cells expressed activity when passaged in serum-free medium. Ten to 15% of AML14 cells grown in serum-containing medium stained positive for γ-glutamyltransferase (data not shown).

Modulation of Serum Protein Expression in AML12 Cells. With continual passage, the AML12 line retained its epithelial morphology and strong expression of many hepatocyte mRNAs (Fig. 3). However, under these conditions the expression of albumin and A1AT mRNAs gradually declined (Fig. 3), and a smaller percentage of cells stained for albumin (Fig. 4*C*). We found that the passage and maintenance of AML12 cells in serum-free medium reversed the decline in the expression of serum protein mRNAs. When cells previously kept for 6 months in 10% FBS were passaged twice in serum-free medium, levels of albumin, A1AT, and transferrin mRNAs increased to 90%, 72%, and 362% of their respective levels in cultured hepatocytes (Fig. 3, compare AML12 −FBS with AML12 p6 cells in standard conditions). One to two passages in serum-free medium dramatically upregulated expression of these transcripts even in very late cultures (data not shown). Eighty to 95% of AML12 cells at >p80 (>18 months) treated in this manner exhibited strong albumin staining (Fig. 4, compare *D* with *C*). Passage in defined medium also increased Cx 26 and Cx 32 mRNA levels (Fig. 3). In addition, AFP mRNA was induced, a phenomenon previously described in primary cultures of mouse hepatocytes (32).

To determine whether the reappearance of albumin expression was due to cell selection or to reversion of expression, we used an immuno-overlay technique (4, 28) to identify and subclone albumin-secreting colonies arising from single AML12 cells. When individual albumin-positive colonies were expanded in FBS-supplemented medium, the heterogeneity in albumin expression persisted: 10–40% of the cells in each subclone continued to produce albumin, and this percentage was increased by passage in serum-free medium. Further, an AML12 colony which was initially negative for albumin was subsequently found to contain albumin-producing cells when expanded (data not shown). Thus it appears that many or all of the AML12 cells retain the ability to produce albumin, but that the actual expression is reversibly modulated during tissue culture.

Expression of mTGFα and EGFR in AML Lines. Both AML12 and AML14 expressed high levels of the 1.0-kb hTGFα mRNA and lower levels of endogenous 4.5-kb mTGFα message at early passages (Figs. 1*D* and 3). In both lines, mTGFα mRNA levels were higher than in isolated hepatocytes, presumably due to induction by hTGFα secreted into the medium (12). Whereas AML12 cells continued to strongly express the transgene, expression gradually dropped in AML14 cells, roughly in parallel with a decline in expression of most hepatocyte-specific mRNAs, while Cx 43 mRNA increased. Placing the cells in serum-free medium (Fig. 3, AML14 −FBS lane) markedly increased transgene expression and Cx 26, Cx 32, albumin, and transferrin mRNAs but did not change A1AT mRNA levels. Exposure of cells to $ZnSO_4$ to induce higher transgene expression did not cause further increases in albumin expression (data not shown).

Expression of the 10-kb and smaller EGFR transcripts was ≈2-fold higher in both AML lines relative to hepatocytes. While the 10-kb EGFR mRNA was present throughout the period of study, levels of the 6.0-kb and smaller transcripts declined in AML14 cells after 6 months in culture (Fig. 3).

DISCUSSION

We have established two hepatocyte lines from the livers of transgenic mice overexpressing TGFα. These lines are non-transformed hepatocytes which have been immortalized without viral oncogenes and have retained their differentiated features after many passages. Several criteria establish their hepatocytic origin and phenotype: (*i*) morphology and presence of peroxisomes and bile canaliculi; (*ii*) expression of mRNAs encoding albumin, A1AT, and transferrin; (*iii*) co-expression of Cx 26 and Cx 32 mRNAs; and (*iv*) presence of exclusively LDH isozyme 5. In addition, both hepatocyte lines express mRNAs for specific peroxisomal enzymes (J. Reddy, personal communication).

In the AML12 line, expression of the transgene and mTGFα was stable, and strong expression of Cx 26 and Cx 32 mRNAs was unconditionally retained; high expression of all hepatocyte markers examined was seen in cultures maintained over 1.5 years, after passage in serum-free medium. In contrast to AML12, AML14 lost most of its differentiated characteristics by 14 months (p61), in parallel with the extinction of TGFα expression.

Our analysis of AML12 subclones indicates that the majority of AML12 cells are capable of producing albumin but that expression is modulated by culture conditions. The mechanisms regulating liver-specific gene expression in AML cells are unknown but are likely to involve transcriptional/posttranscriptional events responsive to serum components, growth rate, and intercellular contacts, as observed in other hepatocyte cultures (2, 3, 25, 33).

We could not establish a clear relationship between hTGFα expression and the maintenance of hepatocyte differentiation in culture. In the seven lines initially screened, there was a correlation between transgene and albumin mRNA levels. AML12 continued to express both hTGFα and liver-specific genes, but in AML14 the expression of hepatocytic traits declined along with TGFα expression. Inducing the transgene did not fully restore expression of all hepatocyte markers, so that by 6 months in culture, TGFα production in AML14 was not tightly coupled to differentiation. However, the fact that AML14 no longer needed TGFα to proliferate suggests that additional alterations probably occurred during culture.

In the past, obtaining stable differentiated hepatocyte lines has proven difficult without resorting to transforming techniques. Hepatocytes immortalized with simian virus 40 T antigen can give rise to well-differentiated lines but often

become tumorigenic with continued passaging (3, 4). Lines of liver epithelial (oval) cells have been developed which may serve as hepatocyte progenitors but differ morphologically from mature hepatocytes (29, 34–36). Recently it has been shown that human liver epithelial cells immortalized with simian virus 40 T antigen can express xenobiotic drug-metabolizing enzymes (37). However, in these cases the viral antigen has the potential to complex with the products of tumor-suppressor genes (38). We show here that hepatocytes overexpressing TGFα can be maintained as replicating, untransformed, differentiated lines under standard culture conditions without the introduction of viral oncogenes. In addition to serving as models for the study of tissue-specific gene regulation and hepatocarcinogenesis, both AML12 and AML14 are transfectable (unpublished data) and can thus be used to directly assess the actions of putative growth-regulatory genes.

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