Persistence of Liver-specific Messenger RNA in Cultured Hepatocytes: Different Regulatory Events for Different Genes

H. Isom,* I. Georgoff,* M. Salditt-Georgieff, and J. E. Darnell, Jr.

*Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033; and The Rockefeller University, New York 10021

Abstract. Normal adult rat hepatocytes plated on rat tail collagen-coated dishes and fed a chemically defined medium supplemented with epidermal growth factor and dimethylsulfoxide (DMSO) were examined over a 40-d culture period for (*a*) the amount of albumin secreted; (*b*) steady-state albumin mRNA levels; (*c*) steady-state mRNA levels for six other liver-specific genes and three common genes; and (*d*) transcription of several liver-specific and common genes using isolated nuclei. DMSO-treated hepatocytes in culture for 40 d expressed albumin mRNA at 45% the level of normal liver and five other liver-specific genes at levels ranging from 21% to 72% of those in normal liver. The rate of synthesis of ligandin RNA using nuclei from 40-d hepatocytes in a nascent chain extension assay was 130% of the value obtained for normal liver, indicating that liverlike transcriptional activity for ligandin was maintained in this in vitro culture sys-

tem. In contrast, the rates of synthesis of albumin and phosphoenolpyruvate carboxykinase (PepCK) mRNAs using nuclei from 40-d hepatocytes were 8% and <1%, respectively, and, therefore, were at levels that were much lower than was expected given the steady-state mRNA levels for these two genes. The discrepancy between the steady-state mRNA levels and rates of synthesis of RNA was analyzed, and the results suggest that the albumin and PepCK mRNAs from hepatocytes in culture may be more stable than those from liver. A plateau period for secretion of albumin, expression of albumin, α_1-antitrypsin, ligandin, phenylalanine hydroxylase, and PepCK mRNAs, and synthesis of albumin RNA using isolated nuclei was observed from days 6 to 40. The usefulness at a biological and molecular level of a hepatocyte culture system in which liver-specific genes are expressed over a long plateau period is discussed.

S INCE the time of Harrison (1908), it has been the goal of many developmental biologists to achieve proper differentiation or to maintain differentiation in organ explants or cells in culture. Differentiation can be measured at several different levels, including morphology and the qualitative and quantitative expression of tissue-specific proteins and mRNAs for these proteins. More recently, differentiation also has been measured by determining whether the molecular mechanisms by which tissue-specific gene products are expressed in vivo can be maintained in culture (Clayton and Darnell, 1983; Jefferson et al., 1984; Clayton et al., 1985; Fraslin et al., 1985; Jefferson et al., 1985; Fujita et al., 1987).

Liver and hepatocytes are a favorable choice for studying expression of tissue-specific functions because the number of protein products that are known to be expressed solely or predominantly by liver and have been characterized is greater than for any other cell type. cDNA clones to a wide variety of liver-specific genes have been prepared, and it has been demonstrated that the major basis for liver-specific mRNA production in liver is transcriptional control (Derman et al., 1981). Although the techniques for isolation of hepatocytes from liver have been available for almost 20 years (Berry and Friend, 1969), success at maintaining

differentiated hepatocytes in culture has only been achieved recently. Maintenance of differentiated hepatocytes in vitro has been accomplished by culturing the cells in serum-free hormonally defined medium (Enat et al., 1984); by maintaining cells in a dimethylsulfoxide (DMSO)-supplemented defined medium (Isom et al., 1985); by using specific attachment surfaces, including extracellular matrix and components of matrix (Fujita et al., 1987); and by cocultivating primary hepatocytes with rat liver epithelial cells (Guguen-Guillouzo et al., 1983). When hepatocytes are cultured in serum-supplemented standard tissue culture medium, hepatocyte morphology and production of liver-specific proteins are rapidly lost, the levels of expression of liver-specific mRNAs drop steadily within 5 d in culture and the rates of transcription of liver-specific genes are between 1% and 10% that of liver by 24 h after plating (Clayton and Darnell, 1983). When hepatocytes are maintained in a serum-free hormonally defined medium, albumin and α_1-antitrypsin $(A_1AT)^1$ mRNAs are expressed at least through 5 d in cul-

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ture at levels approaching those of normal liver, but the rates of transcription of these two liver-specific genes are only 3–10% of those in liver (Jefferson et al., 1984). Rates of transcription of albumin at levels 20–100% of those of freshly isolated hepatocytes or intact liver have been reported using nuclei isolated from liver slices in culture for 24 h (Clayton et al., 1985), hepatocytes in coculture with nonparenchymal liver epithelial cells for 8–12 d (Fraslin et al., 1985), or hepatocytes cultured for 5 d in a defined medium supplemented with glycosaminoglycans or proteoglycans (Fujita et al., 1987).

When hepatocytes are plated on collagen-coated plastic cell culture dishes and fed chemically defined medium (CDM) supplemented with DMSO, differentiation is maintained by the hepatocytes for at least 43 d (Isom et al., 1985). Several criteria were used for measuring maintenance of differentiation in DMSO-treated cells, including (a) intact hepatocyte-like morphology at the light microscope and ultrastructural levels, (b) secretion of albumin at the same levels as freshly plated cells throughout the time of culture, and (c) retention of the ability to produce the plasma proteins transferrin and hemopexin. The DMSO system has several advantages for studying regulation of gene expression in primary hepatocytes. First, the cells in the population are reasonably homogeneous in that the presence of DMSO essentially eliminates the natural presence of contaminating nonparenchymal liver cell types and differentiation is maintained without the need to coculture the hepatocytes with a second cell type. Secondly, the system is not particularly complex; the plating surface is rat tail collagen and not matrix or purified matrix components. Finally, and perhaps most importantly, it is the only system in which hepatocytes have been maintained over such a long period of time without loss of differentiation. In this report, analysis of maintenance of differentiation by hepatocytes cultured in DMSO has been extended. Specifically, our objectives were to (a) determine whether the pattern of albumin mRNA expressed by hepatocytes with time in culture corresponded to that previously observed for albumin secretion, (b) examine the hepatocytes with time in culture for the expression of liver-specific mRNAs other than albumin and for common mRNAs, and

(c) determine the rates of transcription of albumin, other liver-specific genes, and common genes using nuclei from long-term hepatocytes.

Materials and Methods

Hepatocyte Cultures

Primary cultures of adult rat hepatocytes were isolated by collagenase perfusion of male Fischer F344 rats (180–200 g) as described previously (Berry and Friend, 1969) and modified (Feldhoff et al., 1977; Isom, 1980). Hepatocytes were washed in RPCD medium (Woodworth et al., 1986) supplemented with 5% fetal calf serum and plated at a density of 10^6 cells per 60-mm plastic cell culture dish that was coated with rat tail collagen (Elsdale and Bard, 1972). RPCD medium contains insulin (0.06 µg/ml), glucagon (0.04 µg/ml), dexamethasone (0.4 µg/ml), and transferrin (100 µg/ml). At 5–6 h after plating, hepatocyte monolayers were fed fresh serum-supplemented RPCD and at 24 h after plating the cells were fed RPCD supplemented with 2% DMSO (Isom et al., 1985) and 25 ng/ml epidermal growth factor (EGF; Collaborative Research, Lexington, MA) (CDM+EGF+DMSO). Hepatocytes were fed fresh CDM+EGF+DMSO every 2 d.

Albumin Secretion

The amount of rat albumin secreted into the culture medium was measured by rocket immunoelectrophoresis as described (Laurell, 1966). Rat albumin (fraction V, Sigma Chemical Co., St. Louis, MO) was diluted in the culture medium and used as a standard. The amount of albumin secreted per cell was determined by dividing the amount of albumin secreted by a culture by the number of cells in the culture. Protein was measured by the method of Lowry et al. (1951).

Recombinant Plasmids

The plasmids used in these experiments are described in Table I. Plasmid DNAs were isolated and purified by previously described methods (Maniatis et al., 1982). The identity of plasmid DNAs was verified by digestion with the appropriate restriction endonucleases followed by electrophoresis in 1.0% agarose gels. DNA concentrations were determined spectrophotometrically.

Extraction of RNA

To isolate RNA from intact liver, minced liver tissue was homogenized in guanidine thiocyanate lysis buffer (6 M guanidine thiocyanate, 5 mM sodium citrate [pH 7.0], 0.1 M β-mercaptoethanol, and 0.5% sarkosyl) (Chirgwin et al., 1979). The cell DNA in the lysate was sheared by repeated passage through an 18-gauge needle. The lysate was then centrifuged through

Table I. Recombinant cDNA-containing Plasmids Used in These Experiments

Abbreviation	Encoded product	Animal source	Plasmid designation	Donor or reference
A11	Albumin	Rat	JB	Sargent et al. (1981)
A_1AT	α_1-Antitrypsin	Mouse	plivS-3	Derman et al. (1981)
Lig	Ligandin	Rat	pGST94	Kalinyak and Taylor (1982)
TF	Transferrin	Mouse	plivS-6	Derman et al. (1981)
PH	Phenylalanine hydrolase	Rat	prPH98	Robson et al. (1982)
PepCK	Phosphoenolpyruvate carboxykinase	Rat	pPCK-2	Yoo-Warren et al. (1983)
A_{2u}	α_{2u}-Globulin	Rat	pSGII	Laperche et al. (1983)
Act	β-Actin	Chicken	pA1	Cleveland et al. (1980)
Tub	α-Tubulin	Human	bα1	Cowan et al. (1983)
CHO-B	Unknown	Chinese hamster	pCHO-B	Harpold et al. (1979)
tRNA	$tRNA^{arg}$	*Xenopus laevis*	pyH48	D. Brown
28S	28S rRNA	Mouse		N. Arnheim

5.7 M cesium chloride and 0.1 M EDTA (Maniatis et al., 1982). The RNA pellet was resuspended, further purified by multiple extractions with phenol/chloroform/isoamyl alcohol (25:24:1), and then precipitated with ethanol and stored at $-20°C$. To isolate RNA from hepatocytes in culture, hepatocyte monolayers in rat tail collagen-coated 60-mm dishes were washed with phosphate-buffered saline (PBS) and the cells from 40-100 plates were lysed directly with guanidine thiocyanate lysis buffer. The lysate was then processed as described for RNA isolated from liver.

Northern and Dot Blot Analysis

For northern blot hybridizations, 15 µg of denatured RNA was loaded into each gel lane and electrophoretically separated on 1.4% agarose gels (Maniatis et al., 1982). RNA concentration was determined spectrophotometrically. Ribosomal RNA concentration on each lane of ethidium bromide stained gels was estimated visually to confirm that the lanes contained equal concentrations of RNA. RNA was transferred to nitrocellulose filters which were baked and prehybridized at 46°C for 48 h in buffer containing 50% deionized formamide, 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% ficoll, 50 mM NaH_2PO_4 (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), and 0.5 mg/ml sonicated salmon sperm DNA. Plasmid DNA was nick translated (Rigby et al., 1977) and denatured in hybridization buffer (50% deionized formamide, 5× SSC, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 20 mM NaH_2PO_4 (pH 6.5), 0.1% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 10% dextran sulfate. Hybridization was performed at 46°C for 48 h with the addition of 1-5 × 10⁶ cpm/ml of specific probe to each hybridization reaction. Filters were washed, dried and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at $-70°C$.

For dot blot hybridization, denatured RNA was diluted in 20× SSC to achieve the desired concentrations and immobilized on prewashed nitrocellulose filters using a Minifold apparatus (Schleicher & Schuell, Inc., Keene, NH). The filters were washed and baked. Dot blots were then prehybridized, hybridized, and washed as described for northern blots. Filters were exposed to preflashed (Laskey and Mills, 1975) Kodak XAR-5 film for autoradiographic detection.

Isolation of Nuclei

Nuclei were isolated and RNA chain elongation to produce nascent-labeled nuclear RNA was carried out as described (Clayton and Darnell, 1983; Clayton et al., 1985). Denatured plasmid DNAs were spotted onto nitrocellulose and hybridized to labeled RNA as previously described (Hofer and Darnell, 1981; Clayton and Darnell, 1983; Clayton et al., 1985). Filters were exposed to preflashed film for autoradiographic detection or dots were cut from filters, placed in liquid scintillation fluid, and counted.

In Vivo Labeling for Analysis of Polyadenylated RNA

Freshly perfused hepatocytes were isolated and immediately labeled with 15 mCi [³H]uridine and 5 mCi [³H]adenosine for 15 min. Cells were then pelleted and total RNA was extracted as described for whole liver RNA extractions. It has been shown in previous experiments with cultured cells (Salditt-Georgieff et al., 1980; Hofer and Darnell, 1981) that the majority of the labeled poly (A⁺) RNA for any gene is nuclear within this period of time. RNA was passaged through an oligo-dT cellulose column and separated into polyadenylated RNA containing (A⁺) and nonpolyadenylated fractions (A⁻).

Results

Morphology of Primary Hepatocytes in Long-Term Culture

Primary rat hepatocytes plated on collagen-coated dishes and fed CDM supplemented with DMSO retain morphologic and biochemical characteristics of normal liver for at least 43 d (Isom et al., 1985). Subsequently, we determined that feeding hepatocytes CDM supplemented with DMSO and 25 ng of EGF/ml (designated CDM+EGF+DMSO) increased the number of cells that retain a hepatocyte-like morphology. At 15 d after plating in CDM+EGF+DMSO, the hepatocytes

were oriented in cords, and mononucleated and multinucleated hepatocytes were observed (Fig. 1 A). We previously reported that in each island of hepatocytes, areas of apparently degenerated cells were observed even when the cells were grown in DMSO-supplemented CDM (Isom et al., 1985). When EGF was included in the medium, the areas of degenerated cells were essentially eliminated. The morphology of individual hepatocytes was similar whether hepatocytes were plated in CDM+DMSO alone or in CDM+EGF+DMSO. By 24 d after plating in CDM+EGF+DMSO, islands were established, even fewer nonparenchymal cells were present, and there was no increase in the number of degenerated areas (Fig. 1 B). The morphology observed at 24 d was maintained through at least 60 d after plating.

Albumin Secretion

We have previously reported that hepatocytes in DMSO-supplemented CDM continue to secrete high levels of albumin, as measured by rocket immunoelectrophoresis for at least 43 d (Isom et al., 1985). When hepatocytes were plated in CDM+EGF+DMSO, the amount of albumin secreted remained high and ranged from 20 to 41 pg of albumin secreted per cell/24 h or 18-40 pg of albumin secreted per nanogram of intracellular protein/24 h; these experiments were carried out by following albumin secretion using a series of parallel cultures isolated from a single animal and harvested at several different time points over a 24-d period.

In experiments in which steady-state levels of RNA were measured or nuclei were isolated to determine transcriptional activity, it was necessary to use the total number of cells obtained from a rat (5 × 10⁷ to 1 × 10⁸ hepatocytes per liver) to prepare sufficient RNA or nuclei for each time point. Therefore, we also carried out experiments measuring albumin secretion with time in culture using cultures from different animals for each time point; in these studies, cultures were harvested over a 40-d period and albumin secretion ranged from 22 to 59 pg per cell/24 h or 13-38 pg per nanogram of intracellular protein/24 h. We concluded that (a) normalization of albumin secretion to cell number or to amount of intracellular protein yielded the same pattern with time in culture, (b) albumin secretion varied to some extent among cultures and from one animal to the next, and (c) the average values for albumin secretion were reasonably constant throughout the long-term culture period. In an independent experiment in which parallel cultures from a single animal were cultured over a 60-d period in CDM+EGF+DMSO, the values for albumin secretion per cell varied from 27 to 52 pg per cell/24 h (Isom and Woodworth, unpublished data).

Northern Blot Analyses of mRNA for Albumin and Other Genes

Hepatocytes maintained in long-term culture were examined for the expression of specific RNAs. Equal amounts of RNA extracted from intact liver and from hepatocytes in culture in CDM+EGF+DMSO for 6, 12, 18, 24, and 40 d were fractionated by size, transferred to nitrocellulose, and analyzed by hybridization to ³²P-labeled cloned DNA. Northern blot analyses of albumin RNA (Fig. 2 A) showed that (a) albumin RNA continued to be expressed throughout the 40-d culture period, (b) the albumin mRNA expressed in hepatocytes in
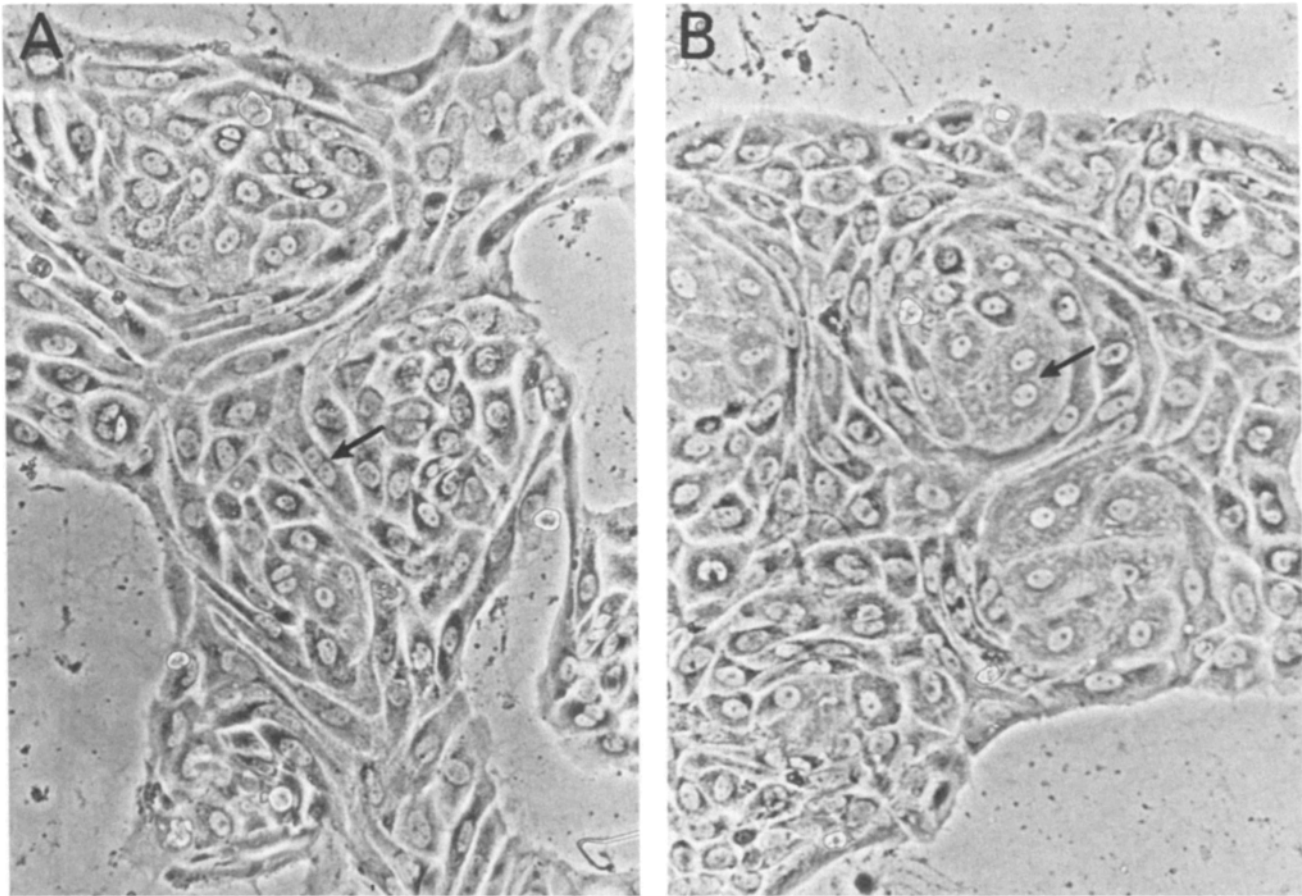
Figure 1. Photomicrographs of hepatocytes in culture. Hepatocytes were plated on rat tail collagen-coated plastic dishes, maintained in CDM+EGF+DMSO and photographed at (*A*) 15 d and (*B*) 24 d after plating. Arrows indicate binucleated cells.

long-term culture was the same size as that found in intact liver, and (*c*) the relative amounts of albumin RNA in hepatocytes in culture for various periods of time remained high, as would have been predicted based on the high amounts of albumin secreted by these cells.
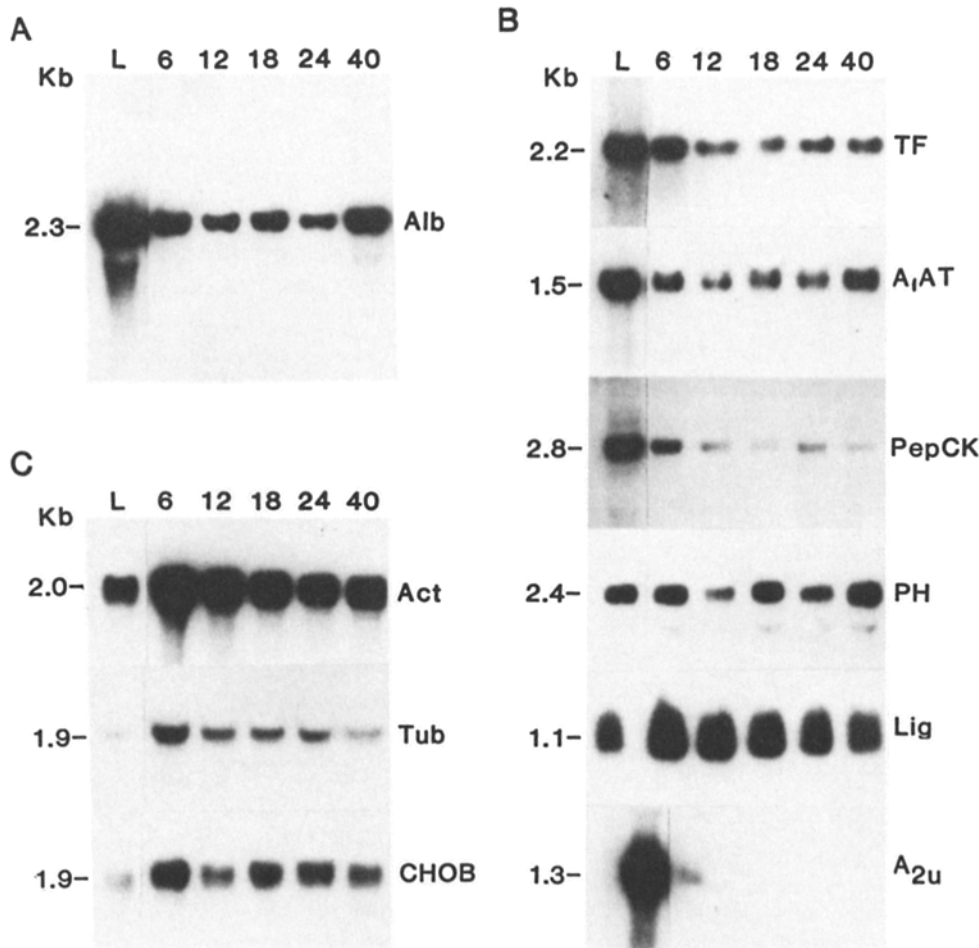
We have previously shown that hepatocytes maintained in CDM+DMSO retain the ability to produce albumin, transferrin, and hemopexin (Isom et al., 1985). In analyzing the use of DMSO to extend the in vitro life span of hepatocytes in culture, we have concentrated on the expression of albumin as a marker of differentiation. Albumin is a plasma protein secreted almost exclusively by hepatocytes in liver and constitutes almost 10% of the protein produced in the liver. In the present experiments, we measured the steady-state levels of albumin RNA in hepatocytes in long-term culture and extended these studies to measure the relative levels of a variety of RNAs (Fig. 2 *B*) expressed only in liver or more abundantly in liver than in other tissues. These included the two serum proteins transferrin and A_1AT; α_{2u}-globulin, a sex hormone–regulated serum protein secreted by hepatocytes from male animals; phosphoenolpyruvate carboxykinase (PepCK), a gluconeogenic enzyme; phenylalanine hydroxylase (PH), the enzyme that converts phenylalanine to tyrosine; and ligandin, one of the forms of the glutathione S-transferase enzymes. We conclude from the northern blot analyses that (*a*) expression of five of the six mRNAs was

maintained throughout the 40-d culture period, (*b*) all six mRNAs present in hepatocytes in culture were similar in size to those detected in the RNA from liver, and (*c*) the level of α_{2u}-globulin message was below the limits of detection by day 12. A quantitative analysis of liver-specific RNAs was performed and will be discussed below.

Northern blot analyses were also used to measure the "common" mRNAs (those mRNAs expressed in most or all cell types) in hepatocytes in long-term culture (Fig. 2 *C*). The common mRNAs examined were those encoding two cytoskeletal proteins actin and tubulin and one mRNA of unknown function found in many cell types and designated Chinese hamster ovary-B (CHO-B) (Harpold et al., 1979). The correct size mRNAs for actin, tubulin, and the CHO-B gene were detected in RNA from hepatocytes in culture for 6, 12, 18, 24, and 40 d. The approximate levels of all three mRNAs in the hepatocytes were equal to or greater than those in whole liver.

Quantitation of Steady-State mRNA Levels of Liver-specific and Common Genes

In addition to the northern blot analyses, the steady-state levels of mRNAs for albumin, other liver-specific genes and common genes were measured quantitatively using dot blot analyses. Different amounts of RNA ranging from 0.05 to 15 µg of liver or hepatocyte RNA were blotted onto nitrocellu-

A

Kb   L   6   12 18 24 40

2.3—   Alb

C

Kb   L   6   12 18 24 40

2.0—   Act

1.9—   Tub

1.9—   CHOB

B

Kb   L   6   12 18 24 40

2.2—   TF

1.5—   A₁AT

2.8—   PepCK

2.4—   PH

1.1—   Lig

1.3—   A₂ᵤ

Figure 2. Northern blot analysis of specific RNA expression in hepatocytes in long-term culture. Hepatocyte cultures were established by in situ perfusion of rat livers and cells were maintained in CDM+EGF+DMSO. RNA was isolated from hepatocytes in culture and from whole liver and 15 µg of total RNA per lane was loaded onto denaturing agarose gels. Lanes are designated as follows: *L*, whole liver RNA; *6*, RNA extracted from hepatocytes 6 d after isolation; *12*, 12 d after isolation; *18*, 18 d after isolation; *24*, 24 d after isolation; and *40*, 40 d after isolation. RNA was transferred to nitrocellulose and hybridized to nick-translated cDNA probes. Each series of bands for a particular species was cut from autoradiographs and compiled for *A*, *B*, and *C*. (*A*) Albumin (pAlb-576). (*B*) Liver-specific RNA species: *TF*, transferrin; *A₁AT*, α₁-antitrypsin; *PepCK*, phosphoenolpyruvate carboxykinase; *PH*, phenylalanine hydroxylase; *Lig*, ligandin; *A₂ᵤ*, α₂ᵤ-globulin. (*C*) Common RNA species: *Act*, actin; *Tub*, tubulin; *CHO-B*, Chinese hamster ovary B.

lose and hybridized to the cDNA probe for albumin and to the other liver-specific and common gene probes that were used for northern blot analyses. Densitometric analyses of autoradiographs of the dot blots were carried out to determine the relative intensities of the hybridizations and, in so doing, the relative steady-state levels of each specific RNA (Table II). The steady-state levels of ligandin and the A₁AT mRNAs in hepatocytes cultured for 40 d were the highest for the liver-specific genes and ranged from 67% to 72% of those in liver. Albumin mRNA levels in hepatocytes at the various times after plating ranged from 43% to 54% of those in liver. PH mRNA was expressed at approximately the same level as albumin mRNA. Although the PepCK mRNA levels were only ∼28% of those in liver, PepCK mRNA was definitely expressed and the ability to express PepCK mRNA at this level was constant from days 6 to 40 (see Discussion). As had been predicted from the northern blots, no α₂ᵤ-globulin was detectable by dot blot analysis of RNA from 12-d cells. The steady-state levels of the common mRNAs were highest on day 6 and, throughout the remainder of the culture period, consistently were equal to or as much as twofold greater than the levels in liver. The levels of actin mRNA in hepatocytes cultured in CDM+EGF+DMSO were two- to fourfold higher than those in liver from day 6 on. Steady-state levels of all common gene transcripts

decreased with time in culture but did not fall significantly below levels in vivo.

Transcriptional Assays for Expression of Liver-specific and Common RNAs

To determine whether the levels of albumin and other liver-specific RNAs expressed in hepatocytes in long-term culture were the result of the transcription rate of these genes, transcriptional assays measuring nascent labeled nuclear RNA were carried out. Nuclei were isolated from intact liver and from hepatocytes cultured in CDM+EGF+DMSO for 40 d and RNA transcripts were elongated in the presence of [α-³²P]UTP. Equal amounts of radioactive RNA from the different samples were hybridized to dots of plasmid DNA on nitrocellulose and subjected to autoradiography. The amount of hybridization was determined by counting the individual dots or by densitometry. Plasmids containing DNA complementary to 28S RNA and to tRNA^arg were included as controls.

When nuclei from liver were used in this assay, the transcriptional signals for albumin and PepCK were significant. The rate of transcription of the albumin gene in nuclei from 40-d hepatocytes was 8% of that in liver (Fig. 3, Table III). The rate of transcription for PepCK in hepatocytes was <1%. Thus, the steady-state levels of mRNAs for albumin (45%)

*Table II. Steady-State RNA Levels in Liver and Isolated Hepatocytes**

| | RNA from hepatocytes | | | |
| | Days in culture | | | |
Probe	6	12	18	40
Alb‡	54	46	43	45
A_1AT	60	58	54	72
Lig	75	91	75	67
TF	76	44	36	21
PH	61	55	40	48
PepCK	28	24	29	28
A_{2u}	11	0	0	0
Act	459	283	319	246
Tub	248	185	149	74
CHO-B	142	122	122	96

Abbreviations in this table are as given in Table I.
* Total RNA extracted from whole liver or from hepatocytes in culture was hybridized to each specific probe. RNA levels were quantitated by densitometric scanning of autoradiographs of dot blot hybridization. Each value was calculated from the equation: (hybridization intensity of hepatocyte RNA/hybridization intensity of liver RNA) × 100.
‡ Alb, albumin, pAlb-576 (J. Taylor).

	A	B	C
1	Al4	Al1	Al0
2	Tf	PepCK	PH
3	Tub	Act	Lig
4	pGEM	pBR322	CHOB
5	Liv-4	A_1AT	Liv-2
6	tRNA		Liv-5
7	28S		

Figure 3. Dot blot autoradiographs for liver-specific and common gene transcription in hepatocytes in long-term culture. Nascent-labeled RNA from nuclei from whole liver (*Liver*) and from hepatocytes on collagen-coated surfaces in CDM+EGF+DMSO for 40 days following isolation (*Day 40*) were prepared and hybridized to dots of cDNAs for liver-specific and common genes. Abbreviations used include: *Al0* (JC, Sargent et al., 1981), 5′ end of albumin gene (noncoding sequences); *Al1* (JB, Sargent et al., 1981), 5′ end of albumin gene (coding sequences); *Al4* (D, Sargent et al., 1981), 3′ end of albumin gene; *TF*, transferrin; *PepCK*, phosphoenolpyruvate carboxykinase; *PH*, phenylalanine hydroxylase; *Tub*, tubulin; *Act*, actin; *Lig*, ligandin; *pGEM*, pGEM-1; *CHO-B*, Chinese hamster ovary B; A_1AT, α_1-antitrypsin; *tRNA*, tRNA$^{\text{arg}}$; *28S*, 28S rRNA.
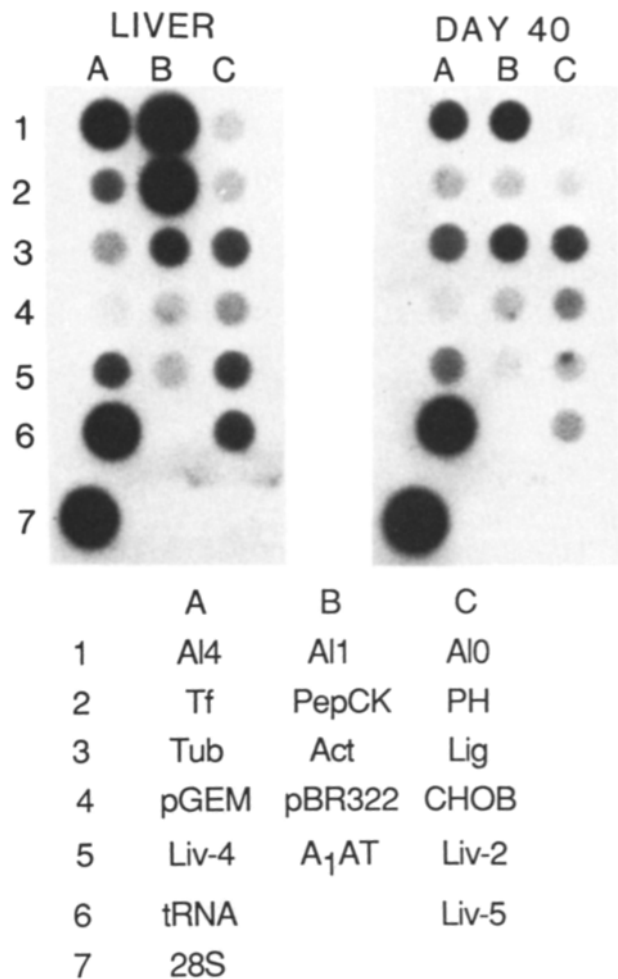
and PepCK (28%) in 40-d hepatocytes were much higher than was expected given the rates of transcription for these genes. The transcriptional signal for ligandin was considerably weaker than for albumin or PepCK, but sufficiently above background to allow quantitation. The rate of transcription of the ligandin gene was 130% of that in liver, indicating that the high steady-state levels of ligandin mRNA in 40-d hepatocytes (67%) did correlate with the rate of transcription for this gene. The results for ligandin show that the rate of transcription of at least one liver-specific gene in hepatocytes in long-term culture is similar to the normal rate in liver. The transcriptional signals for several liver-specific genes were not sufficiently above background to allow a quantitative comparison. In addition, because the nuclei in these studies were isolated from hepatocytes of rat origin, we present in Table III only those results obtained from hybridizations in which the liver-specific gene probes were of rat origin. The transcriptional signal for the common gene actin was approximately equal when nuclei from liver or 40-d hepatocytes was used in the assay.

Effect of Time in Culture on Rate of Transcription of Liver-specific Genes

The low rates of transcription for albumin and PepCK were observed using nuclei isolated from hepatocytes in culture for 40 d. To determine whether the decreased transcriptional activity was dependent upon the length of time hepatocytes were in culture, nuclei were isolated from hepatocytes cultured in CDM+EGF+DMSO for 6 and 24 d, and the transcriptional activity was measured as described for nuclei from 40-d cells. The low rates of transcription for albumin and PepCK were apparent in nuclei from 6-d cells (Table IV).

Percentage of Polyadenylation of Albumin and PepCK Messages from Liver

The ability of hepatocytes in long-term culture to generate large amounts of specific mRNAs suggests that hepatocytes in intact liver compared with hepatocytes in culture (*a*) pro-

duce less stable mRNAs for these genes or (*b*) underutilize nuclear or precursor forms of these gene products. The latter hypothesis was tested by measuring whether extra albumin or PepCK transcripts were made in the liver and not processed. The liver was disaggregated and the cells were immediately radioactively labeled for 20 min at which time the RNA was extracted and poly A⁺ RNA was prepared. The labeled RNA was hybridized to cDNA sequences complementary to albumin and PepCK RNA. In both cases the majority of the RNA complementary to the albumin or PepCK probes was in the poly A⁺ fraction (Table V) indicating that nuclear RNA is efficiently utilized by liver. The inference from these experiments is that the albumin and PepCK primary transcripts are efficiently utilized in hepato-

| | Transcriptional activity* | | |
	Liver	40-d hepatocytes	Activity in hepatocytes‡
	cpm	cpm	%
All	1940	155	8.0
PepCK	1340	14	1.0
Lig	50	68	130.0
Act	85	80	94.0

Abbreviations in this table are as given in Table I.
* Nuclei extracted from liver and from hepatocytes maintained in CDM
+EGF+DMSO for 40 d were used in nascent chain extension assays. cDNA
sequences for All, PepCK, Lig, and Act were spotted to nitrocellulose and
hybridized to labeled RNA generated by nascent chain extension. Filters and
individual dots were counted to determine cpm hybridized. The background
from hybridization to pBR322 was subtracted from each filter.
‡ Percent activity in hepatocytes was determined by the following equations:
(counts per minute hybridized using hepatocyte nuclei/counts per minute hybridized using liver nuclei) × 100.

Table IV. Effect of Time in Culture
on Rate of Transcription

| | Transcriptional activity for nuclei from hepatocytes relative to nuclei from liver | | |
| | Days in culture | | |
Probe	6	24	40
All*	8.7‡	8.6	8.0
PepCK	BD§	BD§	0.6

Abbreviations in this table are as given in Table I.
* cDNA sequences for All and PepCK were spotted to nitrocellulose and
hybridized to labeled RNA generated by nascent chain extension.
‡ Nuclei extracted from liver and from hepatocytes maintained in CDM+
EGF+DMSO for the indicated time period were used in nascent chain extension assays. Relative values for rate of transcription were obtained using the
following equation: (hybridization intensity using hepatocyte nuclei/hybridization intensity using liver nuclei) × 100.
§ BD, below a detectable level.

cytes in vivo at least at the nuclear level. It seems likely,
therefore, that albumin and PepCK mRNAs in hepatocytes
in culture are more stable than the same mRNAs in liver.

Discussion

Primary hepatocytes cultured on rat tail collagen-coated
plates and fed a hormonally supplemented chemically defined medium containing DMSO over a 40-d culture period were successfully used to study gene expression at the
molecular level. The effects of DMSO on long-term culture
of hepatocytes were dramatic in that 40-d-old hepatocytes
continued to express at least six liver-specific genes at levels
ranging from 21% to 72% of those in normal liver and all six
mRNAs were similar in size class to the corresponding
mRNAs expressed in intact liver. The seventh liver-specific
gene examined, α_{2u}-globulin, was only weakly expressed by
day 6 cells and was not detectable in day 12 cells. Loss of
expression of α_{2u}-globulin mRNA by DMSO-treated hepatocytes was not surprising because the hormones necessary
for expression of this gene in vivo were not present in the
culture medium.

Table V. Radioactive Labeling
of Freshly Isolated Hepatocytes to Evaluate
Percent Polyadenylation of Transcripts

| | | | A⁺ binding§ | |
Probe*	A⁺	A⁻	Uncorrected	Corrected
	cpm/fraction‡		%	%
PepCK	2,560	1,420	64	95
All	466	367	56	78

* DNA was blotted to nitrocellulose and hybridized to RNA extracted from
cells prepared and labeled as described in Materials and Methods.
‡ Total labeled cellular RNA was separated into polyadenylated RNA-containing (A⁺) and nonpolyadenylated (A⁻) fractions and the counts per minute/fraction hybridized to each specific cDNA probe was determined by liquid
scintillation counting of nitrocellulose filters containing hybridized RNA.
§ Percent polyadenylated RNA binding was calculated on the basis of cpm
bound to nitrocellulose filters after single passage through oligo-dT cellulose
columns (uncorrected) or adjusted to account for the failure of all polyadenylated RNA to bind to the oligo-dT cellulose column (corrected). Correction
factors were determined by hybridization to both cDNA sequences of polyadenylated RNA derived from the initial A⁻ fractions repassaged through
oligo-dT cellulose.

The three common genes analyzed in this study were actin, tubulin, and CHO-B. It has previously been reported that
the levels of actin and tubulin mRNAs in hepatocytes in culture are markedly higher than in liver (Clayton and Darnell,
1983) and that hepatocytes cultured in hormonally defined
medium supplemented with heparin, dextran sulfate, or carrageenan express lower levels of actin and tubulin mRNAs
than hepatocytes cultured in hormonally defined medium
(Fujita et al., 1987). In the present study, it was readily apparent that although all three genes were expressed by 6-d
hepatocytes at levels higher than those in normal liver, actin
mRNA levels were particularly high relative to tubulin and
CHO-B. Similarly, although the tubulin and CHO-B mRNA
levels in 40-d hepatocytes declined to levels similar to those
in liver, the actin mRNA levels declined but were still more
than twofold greater than those in liver. The elevated expression of actin mRNA was most likely caused by the fact that
the hepatocytes were cultured in the presence of EGF. It has
been previously reported that treatment of quiescent AKR-
2B mouse embryo cells with EGF results in an increase in
actin mRNA levels by 2 h after treatment (Elder et al., 1984).
EGF stimulation has no effect on α-tubulin mRNA levels in
AKR-2B cells.

Although no quantitative data have been previously reported for mRNA levels for ligandin and A_1AT, a comparison of the data presented here with the northern blot
analyses previously published indicates that the levels of expression of ligandin and A_1AT mRNAs in DMSO-treated
hepatocytes maintained throughout a 40-d culture period
were greater than was observed for hepatocytes in hormonally defined medium for 5 d and greater than or equal
to the levels from hepatocytes in hormonally defined medium
supplemented with carrageenan (Jefferson et al., 1985; Fujita et al., 1987). The data reported in this study represent
the first evidence of maintenance of expression of PH,
PepCK, and transferrin mRNAs in hepatocytes in long-term
culture. In examining the values that have been obtained for
the steady-state levels of PepCK mRNA and transcription of
PepCK RNA from isolated nuclei, it is important to realize
that CDM contains glucagon, dexamethasone, and insulin,

and that these three substances regulate PepCK activity (Barnett and Wicks, 1971; Gunn et al., 1975). To examine expression of PepCK mRNA in hepatocytes in long-term culture in more detail, it will be necessary to test the effect of altering the hormonal content of the medium on expression of this gene.

Transcription of albumin RNA from isolated nuclei by nascent chain extension assay was only 8% of that found using nuclei from normal liver. Inasmuch as albumin mRNA was expressed at 45% of that in normal liver, the maintenance of expression of high levels of albumin RNA in DMSO-treated hepatocytes is not accounted for by its transcription; that is, the transcription of albumin RNA was only 18% of what it should have been to account for the amount of albumin mRNA made. Similarly, transcription of PepCK was only 1% of that in normal liver whereas PepCK mRNA levels were 28% of those in liver. Analysis of nuclei from hepatocytes in culture for 6, 24, and 40 d showed that the rate of synthesis of albumin RNA was already apparent by the time the cells had been in culture for 6 d and was maintained throughout the culture period. In fact, the rates of albumin RNA synthesis for nuclei taken from cells at the three different time points were remarkably similar. It has been shown previously (Jefferson et al., 1984) that hepatocytes cultured in hormonally defined medium for 5 d express high levels of albumin mRNA but the transcription of albumin RNA, as measured by nascent chain extension assay, was considerably less than normal liver. We conclude from this previous study and our current study (a) that both steady-state mRNA and transcriptional analyses need to be carried out to evaluate specific cell culture conditions and (b) that the mechanism by which steady-state albumin mRNA levels are achieved in hepatocytes in culture differs from the mechanism used in vivo. It therefore seems that providing hepatocytes in vitro with specialized medium and culture conditions that enable the cells to appear differentiated using steady-state albumin mRNA levels as the criterion for differentiation does not necessarily mean that the cells are as differentiated as normal liver if the criterion for differentiation is that the same mechanism of achieving albumin mRNA levels is functioning in vitro and in vivo.

The rate of synthesis of ligandin RNA from isolated nuclei by nascent chain extension assay was 130% of that found using nuclei from normal liver. This observation contrasted strongly with what was observed for synthesis of albumin and PepCK. It is possible that subtle changes in culture conditions may be needed to idealize any one hepatocyte system for expression of a specific gene. For example, it has been previously demonstrated that dexamethasone in the medium affects the rate of synthesis of collagen, fibronectin, and tyrosine aminotransferase RNAs but has no effect on the rate of synthesis of other liver-specific genes (Jefferson et al., 1985). It appears that CDM+EGF+DMSO is an ideal medium for maintaining expression of the liver-specific gene ligandin by hepatocytes in culture. In previous studies, hepatocyte mRNAs have been divided into two groups, the common and liver-specific genes. The findings with ligandin, albumin, and PepCK in this study indicate that (a) the decrease in rate of synthesis of albumin and PepCK in hepatocytes in culture is not caused by the loss of a single necessary transcription factor involved in the synthesis of all liver-specific genes, and (b) a variety of different molecular mechanisms may function

in the maintenance of expression of individual liver-specific genes. Hepatocytes cultured in CDM+EGF+DMSO can be used to test the effects of adding defined agents on the transcription of specific genes.

Regulation of the amount of specific mRNA molecules has been demonstrated at the levels of synthesis, RNA processing, and mRNA stability (Darnell, 1982). A discrepancy exists between the synthesis of albumin and PepCK RNAs and the steady-state levels of the mRNAs for these genes in DMSO-treated hepatocytes in culture. One possible explanation, that liver is less efficient than hepatocytes in long-term culture in polyadenylating albumin and PepCK RNAs, seems unlikely in that we have demonstrated that hepatocytes examined immediately after removal from the liver polyadenylated the majority of the RNA complementary to the albumin or PepCK probes. The most likely explanation for the discrepancy is that the albumin and PepCK mRNAs from hepatocytes in long-term culture are more stable.

This molecular analysis of hepatocytes plated on collagen-coated dishes and fed CDM+EGF+DMSO over a 40-d culture period has extended our knowledge of the DMSO-treated hepatocyte culture system. The plateau of maintenance of secretion of albumin that was described previously (Isom et al., 1985) was also observed at the level of albumin mRNA. The plateau period was examined in the current study from days 6 to 40. We knew from our previous work that the amount of albumin secreted per cell was high, but there was no way to quantitate albumin secretion relative to liver because the amount of albumin secreted by a hepatocyte in the intact liver cannot be measured. Using mRNA levels made it possible to quantitate the amount of albumin mRNA in hepatocytes in culture relative to intact liver. We have also found that the plateau period exists not only for albumin but also for ligandin, A_1AT, PH, and PepCK steady-state mRNA levels. In addition, not only are the steady-state levels of albumin RNA consistent from days 6 to 40, but synthesis of albumin RNA is also constant over at least this time period. In many short-term hepatocyte culture systems, the cells are either recovering from the effects of cell isolation or they are degenerating as a result of time in culture and there is no plateau. The DMSO-treated hepatocyte culture system has a plateau period and can be used at the biological level as an excellent in vitro model system for testing the effects of drugs and chemicals on normal liver function. In addition, the introduction of recombinant DNA sequences into DMSO-treated hepatocytes during the plateau period can be used to test the function of deleted gene segments.

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