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Changes in Liver-Specific Compared to Common Gene Transcription During Primary Culture of Mouse Hepatocytes

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Liver-specific mRNA sequences were examined in primary cultures of mouse hepatocytes. After cell disaggregation by collagenase treatment and for at least 24 h in culture, little change in liver-specific mRNA concentrations was noted. Gradually over a period of 140 h, liver-specific mRNAs declined. In contrast, transcriptional assays in which liver cell nuclei were used to produce ^{32}P-labeled nuclear RNA showed that liver-specific gene transcription was greatly diminished within 24 h, while polymerase II transcription of "common" genes and transcription of tRNA and rRNA did not decline. Thus, a prompt differential transcriptional effect seems to underlie the gradual loss of tissue specificity of the primary cultures.

At least two major areas of ignorance exist about the molecular basis for tissue-specific gene control. First, what are the signals that are responsible for tissue specificity? Second, how do these signals, once transmitted and received, result in specific gene expression? If a direct biochemical approach (including molecular genetics) is to be successful in answering these questions, then a ready supply of a single type of differentiated cell would be, if not a necessity, a distinct advantage.

The liver has long been recognized as a favorable choice for the study of tissue-specific gene functions (8). Many proteins are synthesized only (or mainly) by hepatocytes, and over one-half of the total cell nuclei and up to 90% of the mass of the liver are a single cell type (9). In addition, many hepatocytes are tetraploid so that the mass of DNA from which RNA synthesis takes place is probably 75% in hepatocytes. Recently, cDNA clones to liver-specific mRNAs have been prepared in a number of laboratories (4, 14). With a collection of purified DNA samples it has been shown that transcriptional control is the major basis for liver-specific mRNA production (4).

Two general ideas dominate considerations about the signals that could maintain transcriptional control in differentiated cells such as hepatocytes. First, circulating substances including hormones, nutrients, and toxic products have been proven to be important in certain cases (5, 16, 18–21a, 24, 26). Second, cell-cell (protein-protein?) contact that is first established during organogenesis (6, 12) in some way provides a stimulus to specific nuclear transcription. The critical cell-cell interaction may occur only during early development or may be established and continue in some form into adulthood. Both the possibility of circulating factors and of cell-cell contact might obviously be tested if functional liver cells could be placed in culture. To this end many years of research have been devoted to obtaining functional cultured liver cells, but reports of success have been varied (15–21a). Cultured liver cells survive and some continued synthesis of liver-specific proteins occurs, but no protocol for long-term maintenance of function is generally agreed upon (21). To determine what steps in specific function are lost during the culture of liver cells, we examined both the concentration of liver-specific mRNAs and the nuclear transcription of liver-specific genes compared with "common" genes, those transcribed by many cell types including undifferentiated cultured cells.

As might have been predicted, knowing that liver-specific protein synthesis often declines during the first week of culture, we found that the concentration of the great majority of liver-specific mRNAs declined with time in culture. The probable explanation for this decline in mRNA content was a dramatic and sudden and decrease in liver-specific gene transcription that occurred soon after the hepatocytes were dispersed by proteolytic action and allowed to attach to a solid substrate. In contrast to liver-specific gene transcription, general protein and RNA synthesis rates and specific synthesis rates for rRNA and tRNA remained high. In addition, specific transcription by RNA polymerase II of common genes, including those producing mRNAs for actin and α- and B-tubulin and three other mRNAs from cultured CHO cells, contin-

ued at a normal rate. These results form the basis for further experiments that may determine whether cell-cell contact or circulating factors or both might be responsible for maintaining the differential transcription that is characteristic of liver cell function.

MATERIALS AND METHODS

Isolation of hepatocytes. Adult male c57/B6 mice (Charles River Breeding Laboratories, Inc.) were injected intraperitoneally with pentobarbital and heparin. After an alcohol wash, a midline ventral incision was made with sterile scissors, and a sterile Teflon cannula was inserted through the right ventricle into the inferior vena cava. The portal vein was cut as perfusion with a sterile wash solution at 37°C was begun (per liter: 400 mg of Kcl, 60 mg of KH_2PO_4, 8,000 mg of NaCl, 47.5 mg of Na_2HPO_4, 1,000 mg of dextrose, and 170 mg of phenol red; 10 mM EDTA; 100 U each of penicillin and streptomycin per ml [GIBCO Laboratories]). A ligature around the inferior vena cava below the liver was tightened, and the flow rate was increased to ca. 7 ml/min and continued for a total of 4 min. The wash solution was then replaced by a prewarmed and filtered collagenase solution (100 mg of collagenase per ml [Worthington Class II, Worthington Diagnostics] and 100 U each of penicillin and streptomycin per ml in Dulbecco modified essential medium with glucose at 4,500 mg/liter [GIBCO Laboratories]). Perfusion was continued for about 7 min or until 50 ml of the collagenase solution had been used. This procedure was carried out under a 40-W lamp, and the liver was maintained at about 30°C.

Perfused livers were cut out, rinsed in warm Dulbecco modified essential medium, and disaggregated with gentle teasing in several batches of the same medium. Before and after centrifugation, cells were counted and tested for viability by staining with 0.45% trypan blue in phosphate-buffered saline. Typically, $>10^8$ total cells were obtained in the original suspension, and ca. 60 to 70% of these were large, nonstaining hepatocytic cells.

Cell culture. The suspension (ca. 50 ml) was spun for 5 min at $50 \times g$ to pellet the hepatocytes. Cells were suspended in an appropriate volume of the culture medium (Dulbecco modified essential medium supplemented with 10 mg each of hydrocortisone succinate, bovine insulin, and inosine [GIBCO Laboratories] per liter; 0.2% bovine serum albumin [Miles Laboratories, Inc.]; 100 U each of penicillin and streptomycin per ml; and 10% fetal bovine serum [Sterile Systems]). Approximately 10^7 viable hepatocytes in 15 or 20 ml of medium were plated in 150-mm Integrid tissue culture plates (Falcon Plastics). Similar results were obtained on 100-mm Nunculon plates (Nunc) by plating proportionately fewer cells on the smaller area. This density was found to be nearly saturating for the attachment of viable cells and resulted in the attachment of few dead cells. The medium was changed at 2 h with a single rinse, allowing for the removal of dead and unattached cells. Most of the viable cells in the original suspension were found to attach within this period of time. The medium was then changed every 1 or 2 days, but none of the results reported here depended significantly upon the feeding schedule. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Labeling of cultures. For protein labeling, the medium from 100-mm cultures was replaced with 3 ml of methionine-free medium (Joklik modification; GIBCO Laboratories) supplemented with 10 μCi of [^{35}S]methionine (New England Nuclear Corp.) per ml and the additives described above. After 30 min, monolayers were washed several times with ice-cold phosphate-buffered saline, scraped into phosphate-buffered saline, and sonicated briefly; protein was precipitated from samples in 10% trichloroacetic acid, collected, washed on glass fiber filters, and counted in scintillation fluid. RNA labeling was similar, except that [^{3}H]uridine (New England Nuclear Corp.) was dried under vacuum and added to the complete culture medium at 10 μCi/ml. RNA was bound to DE81 filters, washed several times in 5% dibasic sodium phosphate–20 mM sodium pyrophosphate, rinsed, dried, and counted in scintillant. Total protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories).

Plasmid DNA samples. cDNA-containing plasmid DNA was isolated from bacterial strains as previously described (10). We thank the following individuals for generously providing the respective clones: Shirley Tilghman for mouse albumin and α-fetoprotein (14), Don Brown for *X. laevis* $tRNA^{Met}$ (pXαtmet1) and $tRNA^{Arg}$ (pyH48), Phil Sharp for rat α-tubulin and Nick Cowan for rat β-tubulin, Paul Szabo for chicken cytoplasmic actin, and Norman Arnheim for mouse 28S rRNA.

Isolation and analysis of poly(A)$^+$ RNA. Total RNA from whole-liver homogenates and cultured cells was isolated in a buffer of 50 mM sodium acetate–10 mM EDTA–0.5% sodium dodecyl sulfate (pH 5.1) by three to five phenol-chloroform-isoamyl alcohol (12:12:1) extractions at 65°C, followed by ethanol precipitation. Polyadenylic acid-containing [poly(A)$^+$] RNA was selected on oligodeoxythymidylate columns (Collaborative Research, Inc.) and optical density at 260/280 nm was used to estimate the concentration. RNA was electrophoresed and blotted as described (4). Individually nick-translated plasmid cDNA clones were hybridized to the blots, which were washed and autoradiographed as described (4), with the following exception: after prehybridization, 500,000 cpm of one nick-translated probe per ml, in a hybridization buffer without polyadenylic acid, polycytidylic acid, $NaPO_4$, or formamide (also not used in prehybridizations), was hybridized for 18 h at 65°C.

Transcription rate analysis. Washed cultured cells (two to six 150-mm plates) were scraped and lysed by Dounce homogenization in ice-cold reticulocyte standard buffer with 0.1% Triton X-100. Whole liver nuclei were prepared from one or two mice as described (4). "Cold perfused" liver nuclei were prepared by a 30-s perfusion with an ice-cold wash buffer (see above) of livers from pentobarbital- and heparin-treated animals. The liver was then minced and Dounce homogenized in Triton X-100, as were the cultured cells. Nuclei were pelleted by a 3-min centrifugation at 800 to 1,000 $\times g$, washed in cold reaction buffer without label, nucleotides, or creatine kinase, and pelleted as before. Nuclei were suspended at 30°C in a reaction buffer containing: 20 mM Tris (pH 7.9); 20% glycerol; 140 mM KCl; 5 mM $MgCl_2$; 1 mM $MnCl_2$; 14 mM 2-

mercaptoethanol; 1 mM each of ATP, CTP, and GTP; 10 mM phosphocreatine; 100 μg of creatine phosphokinase per ml; and 0.5 to 2 mCi of high-specific-activity [α-^{32}P] UTP (New England Nuclear Corp.) per ml. In a typical reaction, 0.3 ml of this reaction buffer was added to a nuclear pellet of 0.1 to 0.2 ml from 2 × 10^7 to 6 × 10^7 cells with gentle tapping or pipetting. For labeling in the presence of 1 μg of α-amanitin per ml, nuclei were suspended in the cold wash buffer for 10 min with added amanitin, pelleted, and suspended in reaction buffer with added amanitin. After 12 to 15 min at 30°C (with occasional tapping), nuclei were pelleted, and nuclear RNA was extracted as described (23). RNA was precipitated twice from 8-ml samples with 2.5 volumes of ethanol, 0.2 M NaCl, and yeast tRNA carrier. Nitrocellulose filters were prepared and prehybridized as described (11), except that plasmid DNAs were dotted (5 μg each) on sheets of nitrocellulose (Schleicher & Schuell Co.) with a 96-well dot blot manifold, and polycytidylic acid was not used. For hybridization, the precipitated RNA was suspended in 1 to 3 ml of 2× TESS (11) with 0.1 mg of yeast tRNA per ml, 0.002% Ficoll, 0.002% polyvinyl- pyrrolidone, and 0.1% sodium pyrophosphate and hybridized (for 35 to 40 h at 65°C) to filters in plastic tissue culture flasks or in plastic wells under paraffin oil. In each experiment, equal amounts of radioactivity were used for all points to be compared, allowing the determination of the change in proportion of total transcription devoted to a given gene over time. Filters were washed several times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015M sodium citrate) at 65°C. Filters were then digested at 37°C with pancreatic RNase (0.4 μg/ml) and T_1 RNase (10 U/ml) in 2× SSC and finally with proteinase K (50 μg/ml) as described (4). Filters were exposed to Kodak XAR-5 film with intensifying screens at −70°C for autoradiographic detection. Densitometric scanning and integration of tracings were carried out with an X-ray Quick Scan Jr. densitometer (Helena Laboratories; Beaumont, Tex.).

RESULTS

Establishment of cultures. Hepatocytes were isolated by perfusing mouse liver in situ with a buffered solution of a partially purified collagenase preparation. In the course of more than 50 isolations, the yield of viable cells (cells that resisted staining with trypan blue) that had a typical hepatocytic morphology was 4 × 10^7 to 7 × 10^7 cells per liver. Single cells plated in Eagle medium supplemented with insulin, hydrocortisone, and 10% fetal calf serum attached to plastic tissue culture dishes within 2 h. After attachment, the cells flattened and spread, establishing apparent intercellular contacts within the first 24 h. Although the cells continued to extend processes, the cell number was stable for at least 1 week, with no observed mitotic figures or any significant change in the number of nuclei per unit area in the culture dishes (assessed by light microscopy). At least 70% of the cells appeared to be binucleate. Other aspects of the characteristic hepatocytic morphology (15, 16, 24) gradually gave way to a more elongated morphology after 7 to 10 days.

To assess the levels of protein and RNA synthesis, cultures from a single mouse liver were labeled, upon establishment of the culture and 1, 2, and 4 days later, with [^{35}S]methionine or [^{3}H]uridine (Table 1). In addition, the total cell protein per culture dish was determined and remained essentially constant through the first 4 days. The incorporation of [^{35}S]methionine increased shortly after the cells were plated and was similar at 1, 2, and 4 days of culture. [^{3}H]uridine incorporation was lower during the first 24 h and had increased at 2 and 4 days of culture. In other measurements (data not shown), a comparable level of [^{35}S]methionine incorporation was obtained in a 19-day-old culture.

These results suggest that the cells maintain general macromolecular synthetic capacities. Most of the cells remain viable, and no replication is evident, at least within the first several days in culture.

Steady-state mRNA levels. Total cellular RNA was extracted from fresh livers and from cells after various periods in culture, and the fraction of poly(A)$^+$ RNA was isolated by oligodeoxythymidylate chromatography. The presence of specific mRNAs was determined in equal portions of various poly(A)$^+$ RNA samples by the Northern blot procedure (electrophoresis of the RNA, transfer to nitrocellulose, and hybridization to labeled, nick-translated plasmid DNAs containing specific cloned mouse DNA segments [25]). The labeled DNA probes that were used included 12 DNA samples containing sequences from liver-specific mRNAs and 4 DNA samples containing sequences complementary to five mRNAs that are common to a variety of tissues. By the Northern blot assay, the initial liver cell suspensions before plating contained amounts of all liver-specific mRNAs approximately equal to that found in whole livers (Fig. 1). Moreover, the levels of all the liver-specific mRNAs remained high through day 1 or 2 of culture. Small variations between 6 and 24 h in several mRNAs, e.g., pliv-6, have not been further investigated but have been observed in several experiments. Since the mRNA concentration of the cultured cells closely resembled that found in whole livers or in cells just after removal from the animal, the process of perfusion, disaggregation, and attachment in cell culture dishes appeared to select mainly hepatocytes. This agrees with the experience described in the literature in which morphology or the presence of liver-specific proteins was used to judge the nature of the cells that attached to culture dishes.

Continued culture of the hepatocytes, howev-

TABLE 1. Macromolecular synthesis rates and total protein in hepatocyte cultures[a]

Time	Total protein (mg)	[^{3}H]uridine incorporated (cpm)		[^{35}S]methionine incorporated (cpm)	
		Total (avg)	Per mg	Total (avg)	Per mg
2 h	16.5, 13.5 (15.0)[b]	46,070, 41,130 (43,600)	2,910	785,400, 888,600 (837,000)	55,800
1 day	14.3, 12.2 (13.3)	29,700, 34,680 (32,190)	2,420	3,922,200, 4,197,000 (4,059,600)	305,000
2 days	12.8, 11.3 (12.0)	108,600, 102,100 (105,350)	8,750	4,303,800, 4,201,200 (4,252,500)	354,000
4 days	13.5, 12.3 (12.9)	76,200, 63,200 (69,700)	5,400	4,378,800, 4,739,400 (4,559,100)	353,000

[a] A total of 16 replicate cultures were established from a single mouse liver. At the indicated times after plating, duplicates were labeled for 30 min as described in the text. Total protein was determined for the uridine-labeled plates. Data are presented as totals per culture (ca. 3.5×10^6 cells).

[b] Number in parentheses is the average of two cultures.

er, showed that, with the exception of pliv-6, the levels of all liver-specific mRNAs declined detectably within 48 h. Most liver-specific mRNAs continued to disappear over the next several days and became virtually undetectable by day 7. In contrast, at the end of 1 week in culture, the cells contained greater levels of all the common mRNAs and virtually identical levels of pliv-6 compared with freshly dissociated cells or the cells of whole livers. The time course of the described changes varied somewhat with different mRNAs. Some liver-specific mRNAs (pliv-7, pliv-8, and pliv-11) declined quickly and remained thereafter at constant but reduced levels, whereas others (albumin, major urinary protein [MUP], pliv-2 through pliv-5, and pliv-10) showed a steady, continuous decline beginning sometime after day 1. The signal for α-fetoprotein (not shown) was very low even in whole livers and freshly isolated cells, which is not surprising considering that the mature hepatocytes are known to make only small amounts of this protein. Nevertheless, mRNA concentration for α-fetoprotein also appeared to diminish during culture.

Transcription rates. The transcription rates were determined in several experiments for various liver-specific genes and compared with those for a variety of common genes. Nuclei were prepared for in vitro labeling from whole-liver homogenates by conventional high-speed centrifugation through sucrose (4). Also, a modified procedure was used to isolate nuclei from suspensions, cultured cells, and washed liver (tissue briefly perfused in situ with ice-cold buffer lacking collagenase). Nuclei were obtained in all of the latter cases by disrupting the tissue or cells in a Dounce homogenizer in hypotonic buffer (reticulocyte standard buffer) containing Triton X-100. The nuclei were isolated and washed by low-speed centrifugation. Previously initiated RNA chains were elongated in the presence of ^{32}P-labeled UTP and unlabeled CTP, GTP, and ATP. The labeled RNA was isolated and hybridized to plasmid DNAs deposited on dots on nitrocellulose filters (13). In comparing different cell samples, equal amounts of labeled nuclear RNA were used so that differential transcription rates could be assessed (see above). The use of autoradiography allowed the detection and comparison of weak signals that exceeded the pBR322 control by no more than a factor of two, which is difficult if not impossible when assaying hybrids by scintillation counting (4). In addition, the sensitivity of the dot hybridization is substantially better. Although inputs in excess of 5×10^7 cpm of labeled nuclear RNA were necessary to obtain signals when total radioactivity was counted (4), the input of as little as 5×10^6 to 10^7 cpm allowed signal detection in a few days of exposure with the use of the dot technique.

Three sets of autoradiographs are presented. In the first experiment (Fig. 2), after 24 h in culture the transcription of the majority of liver-specific genes declined significantly and remained low during an additional 3 days of culture. Transcription of the MUP genes was already reduced within 2 h after the plating of cells, whereas three of the liver-specific mRNA sequences (pliv-6 and pliv-7 in this experiment and pliv-6, pliv-7, and pliv-8 in the following experiment) showed little decrease at any point. As noted above, the mRNA complementary to pliv-6 maintained a constant level in cultured cells equal to that in normal liver. Two of the liver-specific genes (pliv-5 and pliv-10) showed little change in transcription rate within 24 h as compared with fresh liver, but they did decline by day 4.

In contrast to the transcription of liver-specific mRNA sequences, the transcription of the common genes continued at a similar rate throughout the 4 days. In fact, the transcription

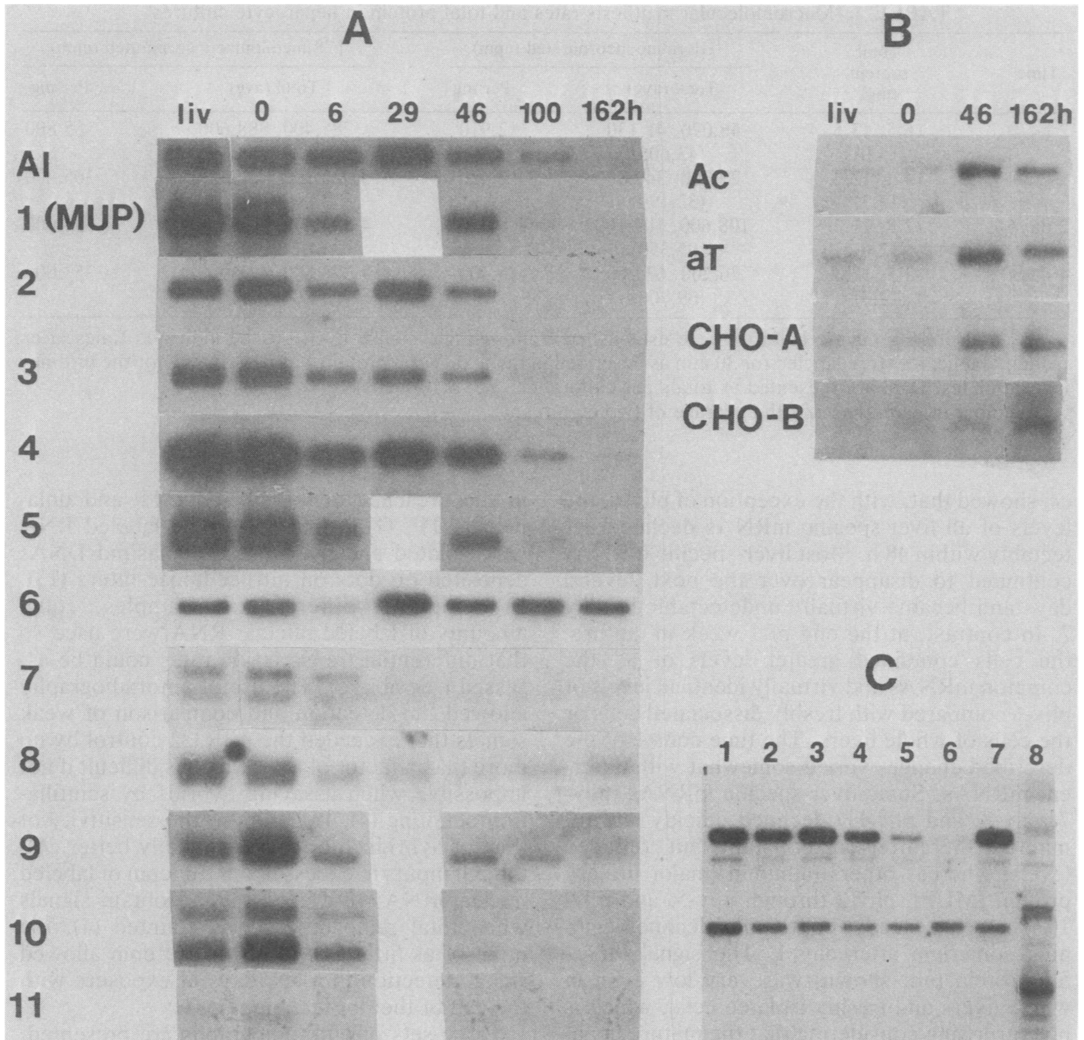

FIG. 1. Northern blot analysis of specific RNA concentrations in hepatocyte cultures through 1 week. Cultures from two mice were established, and RNA from two plates was isolated at the indicated hours after plating. Whole-liver RNA is designated liv, and freshly isolated hepatocytes are 0. Equal amounts of poly(A)$^+$ RNA (measured by optical density), ranging from 0.1 to 1 μg according to the abundance of the mRNA to be measured, were loaded into each well of a denaturing agarose gel, and after electrophoresis the RNA was transferred to nitrocellulose and hybridized to a single nick-translated cDNA probe (two probes in [C]). pBR322 DNA digested by *Hin*cII, *Eco*RI, and *Hin*fI was included on each gel to provide size markers, as shown in (C). Each series of bands for a specific mRNA was cut from autoradiographs and compiled for (A) and (B). (A) Liver-specific RNAs: Al, mouse albumin; MUP, major urinary protein; 2 through 11, the pliv-specific series (4). Blanks represent gel slots where no RNA sample was loaded. (B) Common RNAs: Ac, chicken actin; aT, rat α-tubulin; CHO-A and CHO-B, from CHO cells (10). (C) Photograph of an entire representative blot, hybridized to the albumin probe (upper band) and to a cDNA clone which hybridizes to two mitochondrial RNAs (lower bands). This figure indicates the change in albumin concentration despite the constancy of mitochondrial RNA in the input. Lanes 1 to 6, from 0 h to 162 h (as in A); lane 7, whole-liver RNA; lane 8, pBR322 size markers.

of actin sequences increased dramatically at 2 h before returning to and remaining at normal levels between 24 and 96 h. One other transient variation was noted. The signals for pliv-9, pliv-10, and pliv-11 increased in nuclear RNA from cells just after disaggregation, compared with the transcription in nuclei obtained from whole livers. These changes were apparently reflected in transiently increased steady-state mRNA concentrations (Fig. 1). By 24 h in culture, however, the transcription of these genes had declined. By comparing densitometric tracings of the autoradiographic exposures (Table 2) and by applying various RNA inputs (data not shown), we esti-

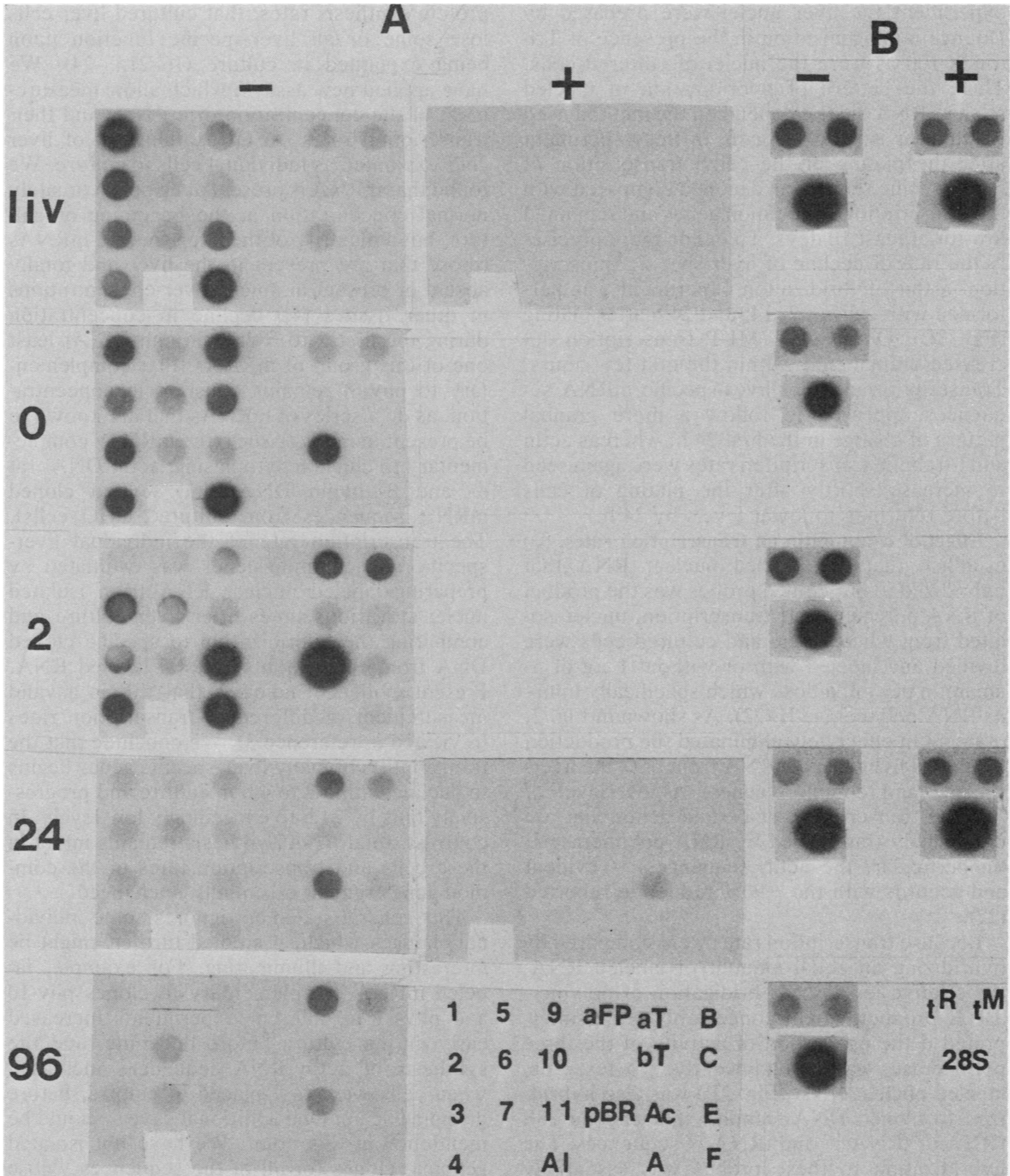

FIG. 2. Dot blot autoradiographs for specific gene transcription rate analysis in hepatocytes. Nascent-labeled nuclear RNA from whole liver (liv), freshly isolated hepatocytes (0), and cultured hepatocytes (at the indicated hours after plating) was prepared and hybridized to dot blots as described in the text. The columns marked + indicate the addition of 1 μg of α-amanitin per ml during the RNA labeling (liver and 24-h samples only). (A) RNA polymerase II products. 1 through 11, Al (albumin), and aFP (α-fetoprotein) are liver-specific RNAs; pBR (pBR322) was used as a measure of nonspecific hybridization; the remainder are common RNAs; A through F, CHO clones; bT, rat B tubulin. Other labels are as described in the legend to in Fig. 1. (B) RNA polymerase I and III products: t^R, $tRNA^{arg}$; t^M, $tRNA^{Met}$; 28S, 28S rRNA.

mate that the decrease in signal intensity between 0 and 96 h for MUP, albumin, pliv-2, pliv-3, pliv-4, and pliv-11 is at least 10-fold and that for pliv-5, pliv-9, and pliv-10 is about 3-fold.

Autoradiographs from a second hybridization experiment are shown in (Fig. 3A). As in the experiment shown in Fig. 2, immediately after isolation the cells continued to synthesize mRNA-specific sequences in the same ratios as did nuclei from whole livers, although in this

experiment the liver nuclei were prepared by Dounce homogenization in the presence of Triton X-100 as were the nuclei of cultured cells. Thus, the pattern of hybridization of labeled nuclear RNA did not depend on the method used for nuclear isolation. Again, in this experiment after the plating of the cells, transcription of liver-specific sequences declined compared with the transcription of common genes and remained low for at least 10 days. To define more precisely the rate of decline of liver-specific transcription, a third hybridization experiment was performed with cells at 7, 14, and 24 h after plating (Fig. 3B). Once again, MUP transcription decreased dramatically within the first few hours. Transcription of other liver-specific mRNA sequences appeared to follow a more gradual pattern of change in the first 24 h, whereas actin and α-tubulin transcription rates were again seen to increase shortly after the plating of cells before returning to lower levels by 24 h.

Effect of α-amanitin on transcription rates. To establish that the labeled nuclear RNA that hybridized to our cloned probes was the product of RNA polymerase II transcription, nuclei isolated from whole livers and cultured cells were divided and labeled with or without 1 μg of α-amanitin per ml, a dose which specifically inhibits RNA polymerase II (22). As shown in Fig. 2, α-amanitin effectively eliminated the production of labeled hybridizable RNA from both the liver-specific and common genes. A low level of residual transcription of certain genes that are presumably transcribed by RNA polymerase II (in particular, the actin sequences) is evident and accords with the ~90% reduction reported (22).

Because transcription rates were compared by hybridizing an equal amount of labeled RNA, the relative levels of hybridization of polymerase II products might conceivably be misinterpreted if the proportion of activity of the three polymerases varied significantly. To test this, labeled nuclear RNA (Fig. 2B) was also hybridized to cloned DNA samples that encode 28S rRNA or $tRNA^{Met}$ and $tRNA^{Arg}$ sequences. The hybridization to these probes was essentially constant, with equal input of radioactivity from different samples indicating a constant input of polymerase II product in the various samples. In addition, the transcription of 28S and tRNA sequences was unaffected by α-amanitin (1 μg/ml).

DISCUSSION

Many workers in the past have concluded, by morphological and histochemical criteria and in a few cases by quantitative measurements of protein synthesis rates, that cultured liver cells lose some or all liver-specific function upon being explanted in culture (16–21a, 24). We have applied new assays, which allow measurement of the concentration of mRNAs and their transcription rates, to an examination of liver cells explanted as individual cells in culture. We found that mRNA is present at an approximately normal concentration at the beginning of culture, but a majority of the liver-specific mRNAs (those that are present in the liver and totally absent or present in much lower concentrations in other tissues [4]) decline in concentration during the first 2 to 5 days in culture. At least one of this group of mRNAs (that complementary to pliv-6) remains constant in concentration, as do a series of mRNAs that are known to be present in many tissues (the mRNAs complementary to chicken cytoplasmic actin DNA, rat α- and β-tubulin DNA, and several cloned mRNA sequences from cultured CHO cells). The transcription rate of the individual liver-specific and common genes was estimated by preparing labeled nuclear RNA from isolated nuclei at various times after liver culture and comparing the hybridization to specific cloned DNA from equal inputs of total labeled RNA. Present evidence indicates that this is a valid measurement of differential transcription rates (reviewed in reference 3). We conclude that the transcription of many liver-specific genes begins to decline within 2 to 4 h in culture and progressively falls by 24 h to exceedingly low levels. In contrast, total RNA synthesis remains intact in these cells and transcription rates of the common genes remain essentially unchanged.

This general statement ignores some individual changes which, if studied further, might be interesting and illuminating. For example, labeled mRNA complementary to clones pliv-10 and pliv-11 in different experiments increased early during culture before declining, and the synthesis of actin RNA sequences increased when cells were first placed in culture, before dropping again. One additional caveat should be mentioned at this point. We have not isolated genomic clones for all of the sequences whose transcription we assayed. We are not certain, therefore, that we were scoring the output from the same genes under different conditions. Likewise, we cannot be sure whether the low levels of transcription that remain are truly meaningful. We have, however, been mainly concerned in this report with a broad view of many genes (over 20 in all) and have reached the central conclusion stated above, i.e., that common gene transcription seems to continue, whereas most liver-specific transcription declines when liver cells are disaggregated and placed in culture. The total transcriptional apparatus has not been

TABLE 2. Changes relative to intact liver in specific gene transcription rates[a]

Probe	Factor change[b] after time in culture:							
	2 h (Fig. 2)	7 h (Fig. 3B)	14 h (Fig. 3B)	24 h				96 h (Fig. 2)
				Fig. 2	Fig. 3A	Fig. 3B	Avg	
Albumin	0	−3	−7	−25	−30	>−50	−50	>−50
1 (MUP)	−20	−25	−50	−20	−50	>−50	−50	−50
2		−2	−6	−10	−50	−50	−37	−30
3				−6	−20		−13	−8
4		−3	−17	−4	−20	−30	−18	−7
5				−2	−4		−3	−3
6				+0.5	−4		−1.5	+0.5
7				−2.5	−6		−4	0
8					−2		−2	
9		+1.5	−2	−2	−30	−10	−14	−4
10				0	−2		−1.5	−3
11	+5	0	−5	−10	−6	−30	−15	−25
Ac	>+50	+7	+6			+2	+2	+2
aT	+6	+3	+2	+2	0	+1.5	+1.5	+2
bT	+5			0	<+2		0	0
A				+4			+4	+4
B	+4			0			0	0
C				0			0	0
E				~0			~0	~0
F				~0			~0	~0

[a] Densitometric scannings of autoradiographs from Fig. 2 and 3 were integrated, and signals from the times indicated were compared with the signal from the intact liver for that particular experiment (a 20-fold decrease below intact liver, e.g., is designated −20). Various exposures of the films were compared to correct for nonlinearity in the autoradiography. In the 2 h column, changes of less than fourfold were omitted for clarity. The averages of the three 24 h points are presented in the column labeled Avg.

[b] Relative to intact liver.

dismantled, but a differential transcriptional effect has resulted.

It is possible that the DNA in the hepatocyte nucleus is still in the same state after disaggregation and culture, but the cells are now deprived of the correct signals necessary for transcriptional control. As discussed above, the signals that are responsible for differentiated function might either consist of some circulating substance or be due to a specific cell-cell association or some other structural feature of tissue-specific organization. Although it is clearly impossible to supply exactly the same amounts and types of nutrients and other circulating materials with which liver cells are normally supplied, we have already found in preliminary experiments that we cannot restore transcription with a mixture of hormones and growth factors. (Increased levels of epidermal growth factor, growth hormone, testosterone, dibutyryl cyclic AMP, and retinoic acid are among the agents tried.) Since all the experiments we report were done in the presence of 10% whole serum, we think it unlikely that restoration of most of the liver-specific transcription could be accomplished simply by additional nutrients.

If this is true, we are left with disaggregation or some other aspect of the transfer of cells to culture as the cause of the transcriptional changes. What possibilities might seem reasonable to consider? At one extreme is the possibility that simple nonspecific damage to all differentiated cells accompanies disaggregation, and as a result of such damage, there is an interruption in specific gene transcription. Even this seemingly trivial explanation, however, demands an adequate mechanistic explanation for the prompt differential effect in gene transcription that we observed. At the other extreme, one can consider that a critical cytoskeletal architecture may exist that is maintained by interactions among successively contacted hepatocytes (and nonparenchymal cells) within a liver lobule. These contacts might be mediated by matrix and cell surface proteins such as collagen, reticular fibers, or cell adhesion molecules (7). The proteins involved may be specific for adult cells or may have been present at the inception of organogenesis and been maintained throughout development. A model system that guides such thinking is the change in protein and nucleic acid synthesis in cultured fibroblasts when deprived of contact with an appropriate surface (2). If any of the liver-specific transcription depends mainly or entirely on cell-cell contact, then we might expect the transcription to be maintained in liver

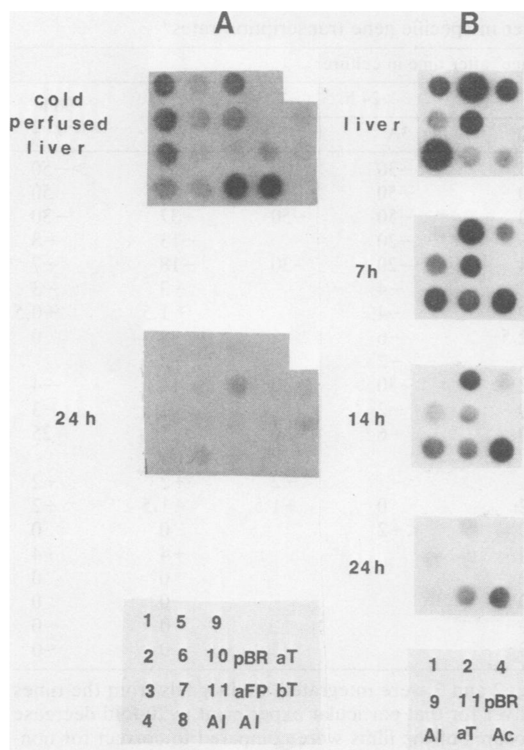

FIG. 3. Dot blot autoradiographs for specific gene transcription analysis in cultured hepatocytes. Nuclei of liver cells were prepared with detergent after perfusion of the liver with cold buffer at 4°C (A). The nuclei of plated cells were prepared in the same manner at the times described in the text. The sample marked liver in (B) was prepared by sedimentation of nuclei through sucrose without detergent (4). Labels of the DNA on each dot are the same as in described in the legend to Fig. 2.

cells that remained in contact, e.g., in slices or in whole perfused livers (1). Tests of these possibilities are under way.

If intact livers could be perfused long enough to demonstrate convincingly that the decreased liver-specific transcription observed in disaggregated cells was a property of disaggregation (and not due to a nutritionally deficient culture medium), then the next set of experiments would concentrate on attempting to restore transcription by allowing reestablishment of cell surface contacts. It should be noted that even in the present experiments, cells that were stretched on the plastic surface of dishes formed membrane contacts, resulting in largely confluent sheets of cells. This did not restore liver-specific transcription, however. There are reports that plating cells on collagen gels and on underlying extracellular matrix material may improve liver-specific functions (19, 21a). In addition, specific adhesion molecules from the liver are known (7). We hope that plating conditions that take these observations into account, together with the ability to measure the most fundamental attribute of cell specificity at any instant in time, will allow a better definition of the conditions required for the maintenance of cell differentiation.

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ADDENDUM

The cDNA-labeled pliv-3 has been identified by sequence analysis to be α1-antitrypsin (B. Citron, K. Krauter, D. Powell, and J. E. Darnell).

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