Hepatocytes in Collagen Sandwich: Evidence for Transcriptional and Translational Regulation

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Abstract. The influence of extracellular matrix configuration on the tissue-specific function of cultured hepatocytes was investigated. Adult rat hepatocytes sandwiched between two layers of collagen gel were compared to cells cultured on a single layer of collagen gel for differences in the total RNA content, the level of albumin-specific mRNA, the rate of albumin gene transcription, and the rate of albumin mRNA translation. Adult hepatocytes in the sandwich system maintained the level of albumin mRNA similar to that found in the normal liver for at least six weeks, whereas the level of albumin mRNA declined rapidly in the single gel system. After one week of culture, hepatocytes in the single gel system could be induced to recover the high level of albumin mRNA and albumin production when a second layer of collagen gel was overlaid at that time. Furthermore, sandwiched hepatocytes maintained significantly higher transcriptional activity compared to cells in the single gel system. In addition to transcriptional control, the ultimate rate of albumin production was shown to depend on the rate of translation, which increased with culture time and reached a plateau in one to two weeks. This increase in translational activity over time in culture was observed in both the sandwich and the single gel systems and, thus, appeared to be independent of the configuration of extracellular matrix.

THE study of tissue-specific gene expression is central to understanding developmental biology. The liver is frequently used in this respect because of its wide range of tissue-specific functions, ability to regenerate in vivo, and responsiveness to changing environments. Since the first introduction of a large-yield preparation of viable cells from the liver (3), hepatocytes have been studied extensively in vitro. The scope of these studies have ranged from gene regulation, metabolism, and intracellular trafficking to carcinogenesis, transplantation, and cryopreservation (1, 7, 11-14, 19, 29, 32, 35, 44, 50, 51). Generally, these studies were performed either on freshly isolated cells or in short-term cultures. This is in part due to a rapid degeneration of hepatocytes in vitro under classical culture conditions. More recently, there have been several methods which prolonged the maintenance of hepatocyte function in culture. These include co-culture with a liver-derived epithelial cell line, addition of complex extracellular matrix, and use of specially formulated culture medium (2, 4, 21, 23, 27, 28, 37, 39, 40, 45, 46, 47, 49). These studies suggest that the state of hepatocytes can be modulated by soluble and insoluble factors, as well as by cellular contact. However, despite much intense effort, there is still a lack of fundamental understanding of all the necessary factors to culture and maintain differentiated hepatocytes.

It is well established that extracellular matrix can modulate gene expression in many biological systems (9, 10, 24–26, 31, 38, 41, 53, 54). It has been suggested that a complex extracellular matrix but not its purified components is necessary to maintained long-term differentiated hepatocytes in vitro (4). However, the complexity and heterogeneity of such biomatrix preparations make further analysis of the mechanism of inducing differentiation difficult. We have previously shown that by sandwiching hepatocytes between two layers of hydrated rat tail tendon collagen gel (15, 16), a variety of hepatocyte functions can be maintained in vitro. This system uses a single, simple, and defined extracellular matrix component and does not require other cell types or specific chemicals. All that appears to be necessary is the culture medium, adequate oxygenation, and the proper configuration of the extracellular matrix. To elucidate the mechanism responsible for the observed pattern of albumin production, the intracellular processes involved with biosynthesis and transport of albumin were analyzed. Since this is a multi-step process which requires precise coordination of many enzymatic reactions, an interruption at any one of these steps conceivably can lead to altered protein production. These steps can be lumped into three general categories: nuclear transcription of the albumin gene, cytoplasmic translation of albumin mRNA, and vesicular transport and exocytosis of synthesized albumin. This report presents evidence which suggests that transcription appears to be responsible for the observed differences in the level of albumin mRNA between the single gel and the sandwich sys-

tems. In addition, the translational activity increases over time in both culture systems to modulate the rate of albumin synthesis.

Materials and Methods

Reagents and Solutions

Type IV Collagenase, lot number 108F-6835, was purchased from Sigma Chemical Co. (St. Louis, MI), Percoll from Pharmacia Fine Chemicals (Piscataway, NJ), DME with 4.5 g/l glucose from Gibco Laboratories (Grand Island, NY), FBS, lot numbers 12103324 and 12103K30, penicillin, and streptomycin from Hazleton (Lenexa, KS), insulin from Squibb (Princeton, NJ), glucagon from Lilly (Indianapolis, IN), EGF from Collaborative Research (Bedford, MA, hydrocortisone from Upjohn (Kalamazoo, MI), and Sea-Kem LE agarose from American Bioanalytical (Natick, MA). Unspecified chemicals were purchased from Sigma Chemical Co., Aldrich (Milwaukee, WI), EM Science (Gibbstown, NJ). Perfusion buffer is 154 mM sodium chloride, 5.6 mM potassium chloride, 5.5 mM glucose, 25 mM sodium bicarbonate, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes), pH 7.4. PBS is 138 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM sodium phosphate, and 1.1 mM potassium phosphate, pH 7.4. 1× HBSS is 138 mM sodium chloride, 5.4 mM potassium chloride, 0.33 mM sodium phosphate, 0.33 mM potassium phosphate, 0.8 mM magnesium sulfate, and 5.6 mM glucose, pH 7.4. Denhardt's solution (1×) is 1% (wt/vol) Ficoll, 1% (wt/vol) polyvinylpyrrolidone, and 1% (wt/vol) BSA. SSC (1×) is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.

Preparation of Hepatocytes

Hepatocytes were isolated from 2–3-mo-old female Lewis rats (Charles River Breeding Laboratories, MA) weighing 180 to 220 g, by a modified procedure of Seglen (48). Animals were anesthetized in a chamber containing saturated ether. The liver, weighing 7 to 8 g, was first perfused through the portal vein in situ with 400 ml of perfusion buffer with 1 mM EDTA at 45 ml/min. The perfusate was equilibrated with 5 liters/min of 95% O_2 and 5% CO_2 through 5 m of silicone tubing (inner diameter 0.058 in, outer diameter 0.077 in) and was maintained by a 100-mm heat exchanger (reflux condenser 283000; Kontes, Vineland, NJ) to be 37°C before entering the liver. The liver was subsequently perfused with 200 ml of 0.05% collagenase in perfusion buffer with 5 mM calcium chloride at the same flow rate. During this time, the liver swelled to about twice the original size. The swollen liver was dissected away from ligaments and the diaphragm and was transferred to a 100-mm dish with 20-ml ice-cold perfusion buffer. The liver capsule was teased apart, and the resulting cell suspension was filtered through two nylon meshes with grid sizes 250 and 62 μm (Small Parts, Miami, FL). The cell pellet was collected by centrifugation at 50 g for 5 min. Cells were further purified by a modified procedure of Kreamer et al. as follows (33). The cell pellet was resuspended to 50 ml, and 12.5 ml of cell suspension was added to 10.8 ml of Percoll and 1.2 ml of 10× HBSS. The mixture was centrifuged at 500 *g* for 5 min, and the cell pellet was washed twice with DME. Routinely, 200 to 300 million cells were isolated with viability between 92 and 99% as judged by Trypan blue exclusion. Nonparenchymal cells, as judged by their size (<10 μm in diameter) and morphology (non-polygonal or stellate), were <1%.

Preparation of Rat Tail Tendon Collagen

Type I collagen was prepared from Lewis rat tail tendons by a modified procedure of Elsdale and Bard (17). Four tendons were dissected from each rat tail and stirred in 200 ml of 3% (vol/vol) acetic acid overnight at 4°C. The solution was filtered through four layers of cheesecloth and centrifuged at 12,000 *g* for 2 h. The supernatant was precipitated with 40 ml of 30% (wt/vol) sodium chloride, and the pellet was collected by centrifugation at 4,000 *g* for 30 min. The pellet was dissolved in 50 ml of 0.6% (vol/vol) acetic acid, and the solution was dialyzed against 500 ml of 1 mM hydrochloric acid five times. For sterilization, 0.15 ml of chloroform was added to the solution. The solution was stirred for two days with a loose cap to allow evaporation of chloroform. A 5-ml aliquot was lyophilized and weighed to determine the yield of collagen. Generally 100 mg was isolated per rat tail. This preparation yields Type I collagen molecules mostly in its native, not cross-linked, triple-helical form (17).

Hepatocyte Culture

Collagen gels were prepared by distributing 1 ml of collagen gel solution (1 part 10× DME, pH 7.4, and nine parts collagen solution at 1.11 mg/ml, chilled on ice, mixed just before use) evenly over a 60-mm tissue culture dish (Falcon, Lincoln Park, NJ) and incubated at 37°C at least 1 h before use. Collagen forms a gel at physiological pH and ionic strength at room temperature, but the rate of gelation is accelerated at higher temperature. Two million viable cells were seeded in 4 ml of medium, consisting of DME supplemented with 10% (vol/vol) FBS, 0.5 U/ml insulin, 7 ng/ml glucagon, 20 ng/ml TGF, 7.5 μg/ml hydrocortisone, 200 U/ml penicillin, and 200 μg/ml streptomycin. This constituted the single gel system. For the sandwich system, an additional 1 ml of collagen gel solution was distributed over the cells after 1 d of culture at 37°C and 10% CO_2. Culture medium was first removed and care was taken to ensure that the second layer of collagen gel was evenly spread over the entire dish. 30 min of incubation at 37°C were allowed for gelation and attachment of the second gel layer before the medium was replaced. Culture medium was changed daily. The collected media samples were stored at 4°C before analyses.

Albumin Assay

Collected media samples were analyzed for rat albumin content by ELISA as previously described (15). Chromatographically purified albumin was purchased from Cappel Laboratories (Cochranville, PA). Antibodies to albumin were purchased from Cappel Laboratories. The 96-well plates (NUNC-Immuno Plate, Maxisorp, Newbury Park, CA) were coated with 100 μl of 50 μg/ml rat albumin in 25 mM carbonate buffer, pH 9.6, overnight at 4°C. The wells were washed four times with PBS plus 0.5% (vol/vol) Tween 20 (PBS-Tween). 50 μl of sample was mixed with equal volume of antibody (800 ng/ml in PBS-Tween) before it was transferred to the wells. After overnight incubation at 4°C, the wells were washed four times with PBS-Tween and were developed with 100 μl of 25 mM citrate and 50 mM phosphate, pH 5, plus 0.4 mg/ml *o*-phenylenediamine and 0.012% (vol/vol) hydrogen peroxide at room temperature. The reaction was stopped with 50 μl of 8 N sulfuric acid after 7 min of incubation. The presence of bound antibodies was detected by the conversion of *o*-phenylenediamine by the conjugated peroxidase. The absorbance was measured at 490 nm with the Dynatech MR600 microplate reader (Chantilly, VA). Positive controls included known concentrations of purified rat albumin, transferrin, and fibrinogen added to culture medium, and negative controls included the culture medium and PBS-Tween. Concentrations of standards were calibrated by their absorbances at 280 nm, using 0.6 as extinction coefficients for 1 mg/ml solution of albumin. Concentrations of samples were determined from a standard curve generated for each ELISA plate. Absolute rates of production were calculated from the concentration by multiplying the total volume of the medium (plus the volume of the collagen gel only on the day when it was introduced to the culture) and dividing by the elapsed time. Results were expressed in μg/h/dish. Generally, two duplicate wells were averaged for each sample, and replicate cultures agreed within 10% of each other.

DNA Assay

DNA content of cultures was analyzed with Hoechst dye 33258 (Calbiochem-Behring Corp., La Jolla, California) as previously described (16). Cultures were rinsed twice with PBS, and 4 ml of 0.05% collagenase solution were added to the dish. After 30 min, of incubation, sheets of cells detached from the dish and were collected by centrifugation. The cell pellets were rinsed twice with PBS and were suspended in 4 ml solution containing 0.1% SDS, 1 mM EDTA, and 0.1 M Tris, pH 7.4. 40 μl of sample was added to 2 ml of solution containing 0.1 μg/ml Hoechst dye, 1 mM EDTA, 2 M NaCl, and 10 mM Tris, pH 7.4. Fluorescence at 365-nm excitation and 458-nm emission was measured on an SLM (Urbana, IL) SPF 500C spectrofluorometer, with lamp voltage set at 1025 V. Calf thymus DNA standard was purchased from Sigma Chemical Co.

Extraction of Total RNA

Total RNA was extracted by a modified procedure of Cathala (8). Cells were lysed with 7 ml of lysis buffer (5 M guanidine thiocyanate) (Fluka, Ronkonkoma, NY), 10 mM EDTA, and 50 mM TRIS, pH 7.5) and 0.6 ml of β-mercaptoethanol. For cultured cells, the lysates from six dishes were transferred with a rubber policeman. For freshly isolated cells, lysis buffer was added to 12 million cells. The lysate was homogenized by Polytron (Du

Pont Instruments, Wilmington, DE) for 45 s, and three volumes of 4 M lithium chloride were added to the homogenate. After overnight precipitation at 4°C, the mixture was centrifuged at 10,000 *g* for 1 h. The pellet was washed with 3 M lithium chloride and was resuspended in 4 ml of TES buffer (10 mM Tris, 1 mM EDTA, 0.2% [wt/vol] SDS, pH 7.5). The suspension was extracted twice with phenol and once with chloroform, followed by a repeated extraction with 4 ml of TES buffer. RNA was precipitated with 0.1 volume of 4 M sodium acetate and 2.2 vol of ethanol at −20°C. The concentration was determined from absorbance at 260 nm, assuming one absorbance unit is equivalent to 40 μg/ml of RNA.

Quantitation of Messenger RNA

Northern blots were used to ascertain the quality of extracted RNA. Hybridization with nick-translated albumin cDNA probe (a gift from Dr. James Darnell, Rockefeller University, New York) yielded a major band at ~2 kbp. For more precise quantitation, duplicates of 0.6, 0.4, and 0.2 μg of total RNA were immobilized onto nitrocellulose filter slots for each sample. The filters were first hybridized with 50% (vol/vol) formamide, 5× SSC, 5× Denhardt's solution, 50 mM sodium phosphate, pH 7, 0.2% (wt/vol) SDS, and 0.2 mg/ml salmon sperm DNA for 4 h at 42°C, followed by hybridization with 4 ml of 50% (vol/vol) formamide, 5× SSC, 5× Denhardt's solution, 40 mM sodium phosphate, 0.1 mg/ml salmon sperm DNA, and 0.1 μg of the labeled albumin cDNA probe at 42°C overnight. The filter was washed with .1× SSC and .1% (wt/vol) SDS at 65°C the next day. Autoradiography was carried out at −80°C with intensifying screens (Lightening Plus, Du Pont Instruments). The radioactivity in each slot was determined by scintillation counting.

Nuclear Run-Off Analysis

Nuclear run-off assays were performed using a modified procedure of Jefferson (30). 20 dishes of cultured cells were first treated with equal volume of 0.4% (wt/vol) collagenase at 4°C for 1 h to digest the collagen gel. Cells were collected by centrifugation at 50 *g* for 5 min. After two washes with PBS, the cell pellet was mixed vigorously in 20 ml of 0.5% (vol/vol) NP-40, 10 mM sodium chloride, 3 mM magnesium chloride, and 10 mM Tris, pH 7.4. The mixture was centrifuged at 300 *g* for 5 min. After another wash with the same buffer, the nuclei pellet was resuspended in 40% (vol/vol) glycerol, 0.1 mM EDTA, 5 mM magnesium chloride, and 10 mM Tris, pH 7.4, and was frozen in liquid nitrogen until nuclei from other samples were ready. Thawed nuclei were washed in 10 ml of 20% (vol/vol) glycerol, 140 mM potassium chloride, 5 mM magnesium chloride, 1 mM manganese chloride, 14 mM β-mercaptoethanol, and 10 mM Tris, pH 8.1. Washed nuclei were resuspended in 180 μl of complete reaction buffer containing 20% (vol/vol) glycerol, 140 mM potassium chloride, 5 mM magnesium chloride, 1 mM manganese chloride, 14 mM β-mercaptoethanol, 1 mM ATP (Pharmacia Fine Chemicals), 1 mM GTP, 1 mM CTP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase (Calbiochem-Behring Corp.) and 20 μl of ^{32}P-UTP (10 mCi/ml, 3000 Ci/mmole, Du Pont Instruments). The mixture was incubated at 30°C for 15 min. Nuclei were lysed with 1 ml of 0.5 M sodium chloride, 50 mM magnesium chloride, 2 mM calcium chloride, and 10 mM Tris, pH 7.4. DNA was digested with 100 U/ml of DNAase I (Worthington, Freehold, New Jersey), and the reaction was stopped with 10 mM EDTA and 0.5% (wt/vol) SDS. The mixture was added to 4.5 ml of 1 mM EDTA, 0.5% (wt/vol) SDS, and 10 mM Tris, pH 8.4, and was extracted twice with 1:1 mixture of phenol/chloroform and once with chloroform. RNA was precipitated with 10 μg/ml of tRNA (Sigma Chemical Co.), 0.2 M sodium acetate, and two volumes of ethanol at −20°C overnight. The pellet was collected by centrifugation at 10,000 *g* for 1 h and was precipitated again with 10% (wt/vol) TCA followed by sodium acetate and ethanol precipitation. The pellet was dissolved in 1 ml of TE (20 mM EDTA, 10 mM Tris, pH 8), and the radioactivity was determined by scintillation counting. Filters with 2 μg per slot of immobilized albumin cDNA probes were first hybridized in 10 ml of 50% (vol/vol) formamide, 5× SSC, 4× Denhardt's solution, 0.1% (wt/vol) SDS, 50 mM sodium phosphate, and 1 mg/ml tRNA at 42°C for 1 d before it was hybridized in 5 ml of 50% (vol/vol) formamide, 5× SSC, 1 × Denhardt's solution, 0.1% (wt/vol) SDS, 50 mM sodium phosphate, 500 μg/ml tRNA, and equal counts of radioactivity (between 4 to 12 million CPM) at 42°C for 2 d. The filters were then washed three times in 1× SSC and 0.1% (wt/vol) SDS for 20 min each and twice in 0.2× SSC and 0.1% SDS for 45 min each. Autoradiography and scintillation counting were the same as for quantitation of mRNA. For the normal liver. 15 to 65 PPM hybridized to the albumin cDNA and 0 to 4 PPM hybridized to the pBR323 cDNA.

Polyribosome Size Analysis

Polyribosome size was estimated by a modified procedure of Lodish (36). 70 dishes of cultured cells were treated with equal volume of 0.4% (wt/vol) collagenase plus 0.1 mg/ml emetine for 1 h at 4°C. The released cells were washed twice with PBS and once with buffer A (10 mM potassium chloride, 1.5 mM magnesium acetate, and 10 mM Hepes, pH 7.5). The cells were resuspended in 2 ml of buffer A and were allowed to swell for 10 min on ice. The cells were dounced 40 strokes with pestle B, and the homogenate was centrifuged at 1,500 *g* for 10 min. The absorbance at 260 nm of the supernatant was adjusted to 30 U, and 1.5 ml of this suspension was mixed with 1 ml of buffer B (240 mM sodium chloride, 7.5 mM magnesium acetate, and 120 mM Tris, pH 8.5), 0.3 ml of 10% (wt/vol) deoxycholate, and 0.3 ml of 10% (wt/vol) polyoxyethylene 20 cetyl ether (Brij 58). 3 ml of this mixture was layered on top of a 34-ml 15–30% (wt/vol) linear sucrose gradient containing 80 mM sodium chloride, 2.5 mM magnesium acetate, and 40 mM Hepes, pH 7.5. The gradient was centrifuged at 26,000 rpm for 90 min in a rotor (SW28; Beckman Instruments, Inc., Palo Alto, CA). The gradient was fractionated by displacement with 50% (wt/vol) sucrose from the bottom of the tube at 1.5 ml/min. The output was monitored at 260 nm continuously, and 4.5-ml fractions were collected in eight tubes, each containing 0.45 ml of 10% (wt/vol) SDS. 1 ml from each tube was diluted with 3 ml of water and 0.4 ml of buffer C (1 M sodium chloride, 10 mM EDTA, and 100 mM Tris, pH 7.5). This mixture was extracted once with 1:1 mixture of phenol/chloroform and once with chloroform. Extraction was repeated with 4 ml of water and 0.4 ml of buffer C. RNA was precipitated twice with 0.1 volume of 4 M sodium acetate and 2 volumes of ethanol. Hybridization condition and scintillation counting were the same as that for quantitation of mRNA.

Pulse-Chase Experiments

Radioactive labeling was performed by a modified procedure of Lee (34). Hepatocytes were cultured on polystyrene dishes for one day under the same condition as described above. The dishes were rinsed twice with methionine-free medium containing the same additives except dialyzed serum was used in place of regular serum. Cells were incubated for 30 min at 37°C in 4 ml of methionine-free medium before the medium was replaced with 1 ml of methionine-free medium with 100 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL). Every 10 min, the medium was collected, and the cells were rinsed twice with PBS before they were lysed with 1 ml of PBS plus 1% (vol/vol) Triton X-100, 0.5% (wt/vol) deoxycholate, 100 KIU/ml aprotinin, 1 μg/ml antipain, 1 μg/ml leupeptin, and 2 mM PMSF. For the chase, the labeling medium was replaced with 1 ml of the usual culture medium, and samples were collected every 10 min. The cellular lysate was mixed overnight at 4°C, and the supernatant was collected the next day by centrifugation at 1,500 *g* for 10 min.

Immunoprecipitation and Gel Electrophoresis

Conditions for immunoprecipitation were pre-determined to quantitatively remove albumin from samples. 5 μl of rabbit anti-human albumin antibody (Accurate, Westbury, NY) were mixed with 25 μl of each sample for 6 h at 4°C before 150 μl of protein A-Sepharose (1:1 mixture of PBS and sepharose gel, Sigma Chemical Co.) was added. After overnight mixing, the pellet was collected by centrifugation for 5 min in a microfuge. The pellet was washed twice with PBS plus 1% Triton X-100, 0.5% (wt/vol) deoxycholate, and 2 mM PMSF before it was resuspended in 105 μl of 10 mM Tris and 1 mM EDTA, pH 8. 60 μl of this suspension was added SDS to 2.5% (wt/vol) and β-mercaptoethanol to 5% (vol/vol). This mixture was boiled for 10 min before bromophenol blue was added to 0.01% (wt/vol). 1 μl of this mixture was applied to a 10–15% (wt/vol) gradient Phast-Gel (Pharmacia Fine Chemicals). The gels were fixed with 25% (vol/vol) isopropanol and 10% (vol/vol) acetic acid for 2 h, followed by two changes of glacial acetic acid for 15 min each. The gels were impregnated with 22% (wt/vol) diphenyloxazole (PPO) in acetic acid for 30 min, and PPO was precipitated with water. The gels were preserved with 5% (vol/vol) glycerol and were dried at room temperature. Autoradiography was performed as described above, except with the omission of the intensifying screens. The autoradiograms were digitized with an Hewlett-Packard Scanjet Plus scanner (San Diego, California), and integrated densities of the bands were determined with the software program IMAGE (National Institutes of Health, Bethesda, MD) on a Macintosh IIfx (Cupertino, California).

Results

Analysis of Albumin mRNA Content in Single Gel and Sandwich Systems

Previously we demonstrated that hepatocytes cultured in the single gel system ceased albumin production after one week of culture, whereas hepatocytes cultured in the sandwich system maintained albumin production for at least six weeks (15). By the end of the first week, the rates of albumin production differed by roughly tenfold although the total DNA contents were similar in these two systems. We first investigated whether this difference in production was reflected in albumin mRNA contents by determining the total RNA content and the level of albumin mRNA in the different systems over time. Total RNA extracted from cells cultured in the single gel system declined over the first two weeks, whereas total RNA extracted from cells cultured in the sandwich system remained fairly constant after an initial decrease (Fig. 1 *a*). A similar pattern was observed for total DNA (Fig. 1 *b*). At the end of the first week of culture, however, the amounts of total RNA and total DNA in both systems are nearly equal. The quantity of RNA per unit of DNA, on the other hand, increased for both systems over the first week and thereafter remained relatively constant (Fig. 1 *c*).

The level of albumin mRNA was found to be high in freshly isolated cells but declined for cells in the single gel system over the first week (Fig. 2 *a*). On the other hand, the level of albumin mRNA in the sandwich system was found to be high at the end of one week. The specific radioactivity (CPM/μg RNA), obtained from the slope of the plot CPM versus microgram of RNA, was determined for each sample. By normalizing the specific radioactivity to that of freshly isolated cells, a comparison was made among cells cultured for different lengths of time (Fig. 2 *b*). The fractional abundance of albumin mRNA in the freshly isolated cells was similar to that found in the normal liver. For hepatocytes in the single gel system, the level of albumin mRNA decreased to roughly half of that in normal liver by the first day and continued to decrease to less than one-tenth of that in normal liver by the end of the first week. In contrast, the level of albumin mRNA in the sandwich system was maintained at a level comparable to that found in the normal liver for at least six weeks of culture.

We had previously shown that hepatocytes cultured for one week in the single system essentially ceased albumin production, but such cells could be "rescued" when a second layer of collagen gel was overlaid (15). It was shown that functions could be restored in rescued cells even after one week of culture in the single gel system. Recovery of albumin production in these rescued cells was accompanied by a parallel change in the level of albumin mRNA (Fig. 3). Thus, albumin production rate was found to correlate with the level of albumin mRNA.

Albumin Gene Transcription

Given that the single gel system and the sandwich system differed primarily in level of albumin mRNA, there should be a concomitant difference in the biosynthesis and/or degradation of albumin mRNA in these two systems. To test the hypothesis that the level of albumin mRNA was controlled transcriptionally, a nuclear run-off assay, which reflected the

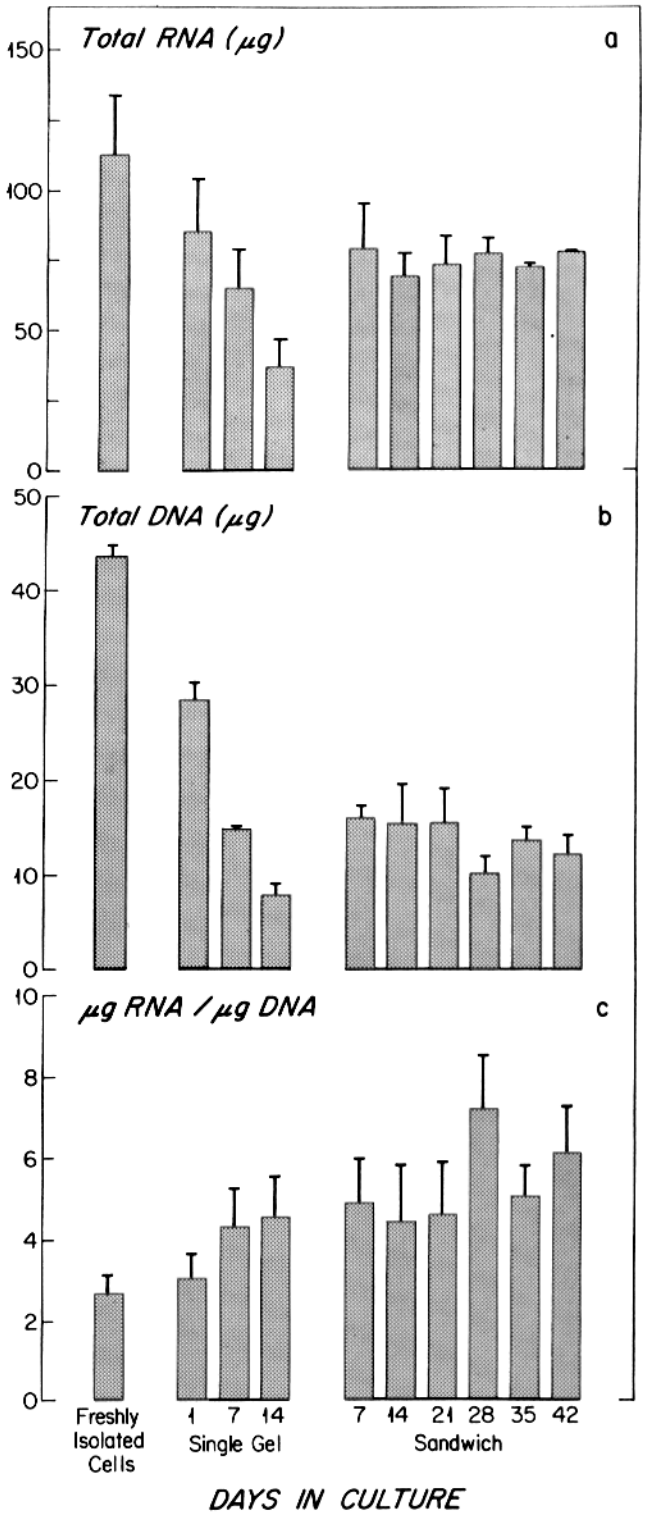

Figure 1. Total RNA and DNA contents of cells cultured in the single gel and the sandwich systems. (*a*) Total RNA per dish decreased to roughly 70% after one week of culture in both systems. Thereafter, total RNA continued to decrease for the single gel system but remained stable for the sandwich system. (*b*) Total DNA content per dish followed similar pattern as the total RNA content. (*c*) The amount of RNA per unit of DNA increased with culture time for both culture systems to roughly twice that of freshly isolated cells by the end of one week. Error bars represent standard deviations of determinations based on four batches of cells.

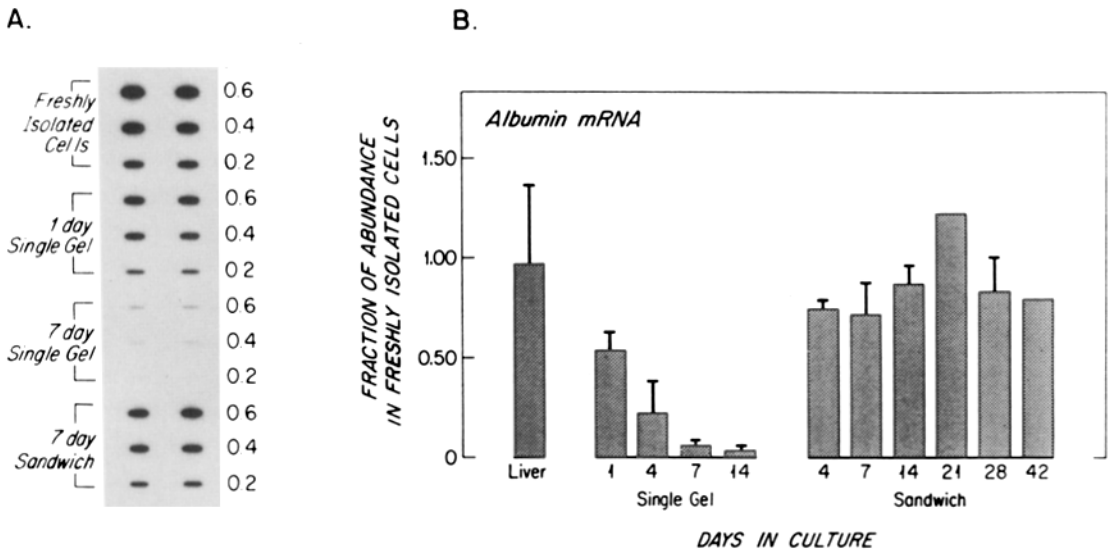

Figure 2. Albumin mRNA level of the single gel and the sandwich systems as measured by slot blot hybridizations. (*a*) Radiolabeled albumin cDNA was hybridized to duplicates of 0.6, 0.4, and 0.2 μg of total RNA for each sample. The level of albumin mRNA appeared to be highest in freshly isolated cells, followed by cells cultured for seven days in the sandwich system and cells cultured for seven days on a single layer of gel. The level of albumin mRNA was barely detectable for cells cultured for seven days on a single layer of gel. (*b*) The specific radioactivity in each sample was normalized to that of freshly isolated cells. For hepatocytes cultured on the single gel, the level of albumin mRNA rapidly decreased over a period of one week. For hepatocytes cultured in the sandwich system, the level of albumin mRNA remained relatively stable for six weeks. The level of albumin mRNA in the intact liver was roughly the same as that in freshly isolated cells. Error bars represent standard deviations from four batches of freshly isolated cells.

number of polymerases transcribing the gene, was used to determine the transcriptional activity in various cell populations. The albumin gene transcriptional activity was found to be high in the liver but was significantly reduced in freshly isolated cells (Fig. 4 *a*). The albumin gene transcriptional activity followed a course of further decline in the single gel system. In contrast, cells cultured in the sandwich system retained higher activity of albumin gene transcription. To obtain a quantitative estimate of the transcriptional activity, the radioactivity of each sample was normalized to that of normal liver. For freshly isolated cells, the albumin gene transcriptional activity was roughly half that of the normal liver (Fig. 4 *b*). After one day of culture in the single gel system, the transcriptional activity decreased to about one-eighth that of the normal liver, and after seven days of culture, the transcriptional activity dropped to less than one-tenth that of the normal liver. In contrast, for hepatocytes cultured in the sandwich system, the transcriptional activity increased to roughly half that of the normal liver and remained steady for at least three weeks. For cells that were first cultured in the single gel system for seven days followed by the addition of a second layer of collagen gel, the transcriptional activity also returned to the level similar to cells cultured in the sandwich system initially (data not shown). These data suggest that the level of albumin mRNA in both the single gel and the sandwich systems correlates well with albumin gene transcriptional activity.

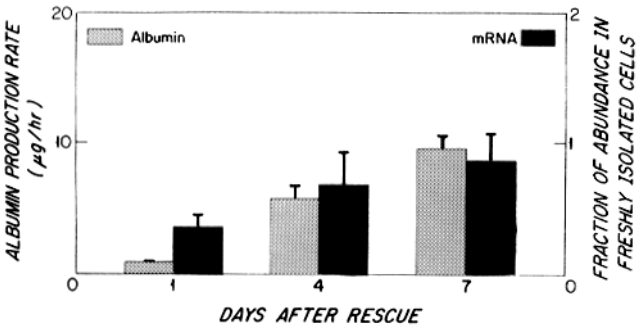

Figure 3. Albumin production rate and albumin mRNA level of hepatocytes cultured for seven days in the single gel system that were subsequently converted to the sandwich system by an additional overlay of collagen gel. Both the level of albumin mRNA and the production of albumin increased over a period one week after the collagen overlay. Results shown represent repeated measurements from a single batch of cells. In repeated experiments, the ultimate level of albumin mRNA recovery varied but correlated with the extent of albumin secretion recovery.

Translation of Albumin mRNA

Previously, we reported that hepatocytes cultured in the sandwich system increased albumin production at least fivefold over the first 1–2 wk before a steady rate was reached (15), even though the total content of DNA was not increased. This suggested that, during the initial culture period, either albumin mRNA was not efficiently used for translation or, alternatively, the synthesized albumin was not processed or secreted properly. The hypothesis that albumin mRNA was not translated efficiently was evaluated with the polyribosome size assay. The distribution of polyribosomes in normal liver is shown in Fig. 5. Each preparation was fractionated into eight aliquots for further analysis of albumin mRNA content. In intact liver, most of the albumin mRNA

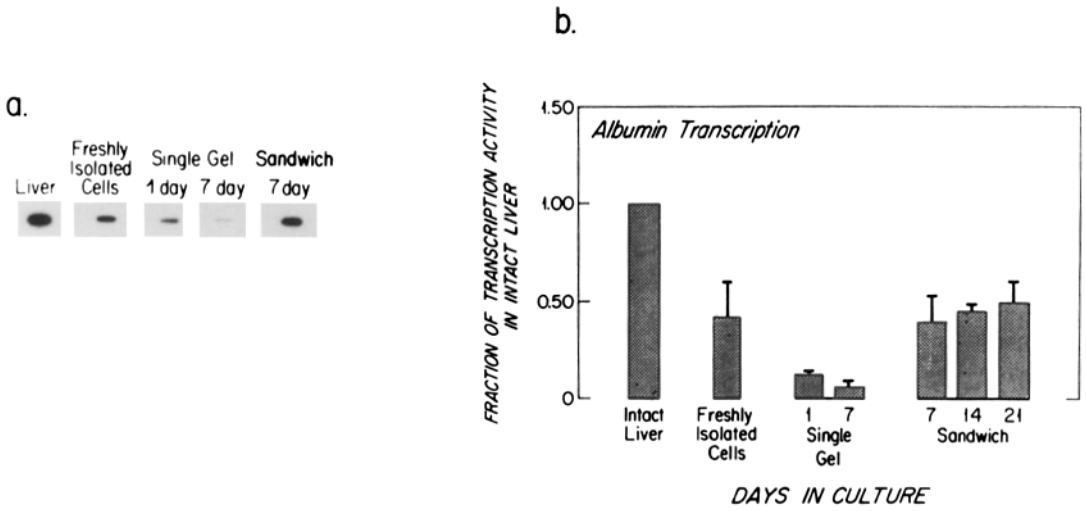

Figure 4. Albumin transcriptional activity of the single gel and the sandwich systems as measured by nuclear run-off assays. (*a*) Transcriptional activity appeared to be highest in the intact liver and was significantly reduced in freshly isolated cells. Transcriptional activity was even lower in cells cultured for one day on a single layer of gel and was barely detectable in cells cultured for seven days on a single layer of gel. In contrast, transcriptional activity was significantly higher in cells cultured for seven days in the sandwich system compared to the single gel system. (*b*) The amount of bound radiolabeled mRNA in each sample was normalized to that of intact liver. For freshly isolated cells, the transcriptional activity was reduced to roughly 40% that of the normal liver. In the single gel system, the transcriptional activity was reduced to roughly 10 and 5% by the first and seventh day, respectively. In the sandwich system, the transcriptional activity remained roughly at 40% for at least three weeks. Error bars represent standard deviations of repeated experiments using four batches of cells.

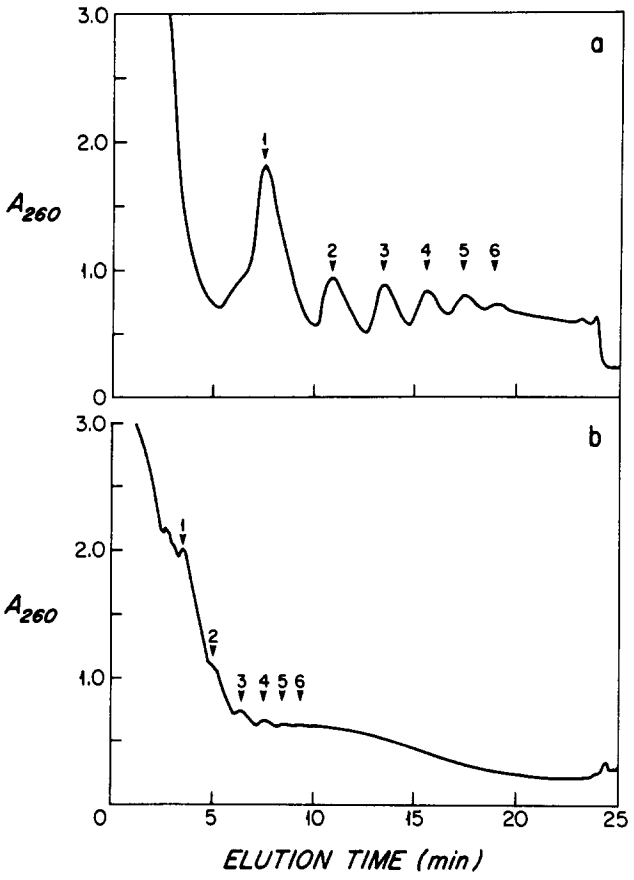

Figure 5. Total polyribosome profiles of the normal liver measured from the absorbance at 260 nm during fractionation of the sucrose gradient. (*a*) Profile was obtained after 3.5 h of centrifugation. Six distinct peaks represent monoribosome, diribosome, triribosome, etc. However, the absorbance did not return to baseline before the end of the sample was reached, suggesting some larger-size polyribosomes were pelleted at the bottom. (*b*) Profile was obtained after 1.5 h of centrifugation. In addition to the previous six peaks, an aggregate which represented larger-size polyribosome was observed.

was found to associate with larger-sized polyribosomes (Fig. 6 *a*). However, for freshly isolated cells, most of the albumin mRNA was associated with smaller-sized polyribosomes. With culture time, there was a gradual increase in the size of polyribosomes associated with albumin mRNA for both single gel and sandwich systems. To obtain a simple quantitative relationship for comparison, the distribution of albumin mRNA was converted to an average polyribosome size by calculations outlined in the Appendix. The average polyribosome size was estimated to be ~10 in normal liver and five for freshly isolated cells (Fig. 6 *b*). With culture time, the albumin mRNA polyribosome size gradually increased to as large as 15. Thus, it appeared that a temporal increase in albumin mRNA translational efficiency occurred for cultured hepatocytes in both systems.

Secretion Kinetics of Albumin

An alternative explanation for the gradual increase of albumin production rate in the sandwich system was an initial malfunction of the secretory process such that only a fraction of the synthesized albumin was secreted. If such a defect were to exist, it should be most dramatic on the first day when the production of albumin was relatively low. To test the hypothesis of defective albumin secretion, pulse-chase

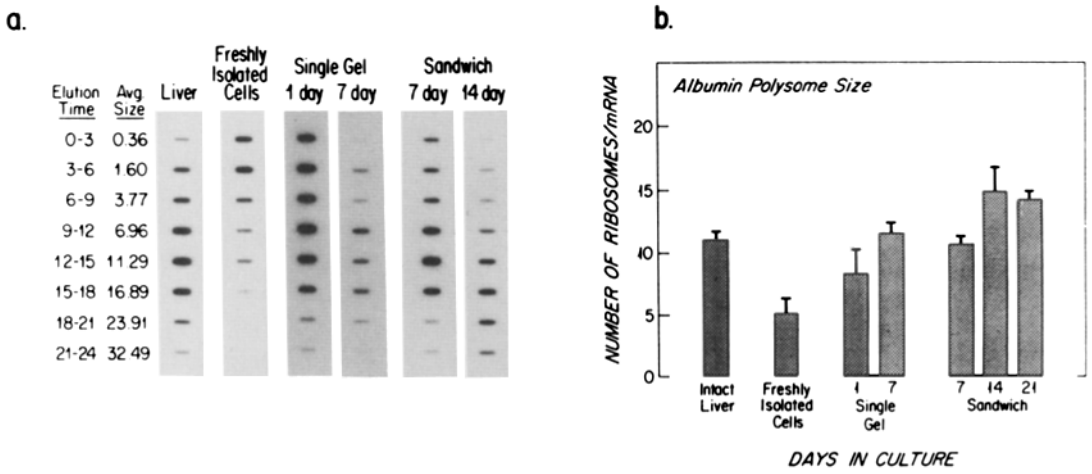

Figure 6. Albumin polyribosome size of the single gel and the sandwich systems as measured by velocity sedimentation through sucrose density gradients. (*a*) The density gradient was divided into eight three-minute aliquots, and each was analyzed for the albumin mRNA content. The mean polyribosome size for each aliquot was calculated by the method outlined in the Appendix. For the liver, albumin mRNA was most abundant in aliquot five corresponding to polyribosomes of size 11. Immediately after cell isolation, albumin mRNA shifted towards smaller size polyribosomes. With culture time, in both single gel and sandwich systems, albumin mRNA gradually shifted towards larger size polyribosomes. (*b*) Average polyribosome size was calculated by the method outlined in the Appendix. The average size of albumin polyribosome was 11 for the intact liver. This was reduced to five for the freshly isolated cells. In the single gel system, the average polyribosome size increased to 8 and 11 after 1 and 7 d of culture, respectively. In the sandwich system, the average polyribosome size increased to 11 and 14 after 7 and 14 d of culture, respectively. No further increase was noted after 21 d of culture. Error bars represent standard deviations from repeated experiments using four batches of cells.

experiments were performed to measure fractional secretion and secretory transit time of albumin. Two compartments were monitored for this purpose: intracellular and extracellular pools. After the administration of radiolabeled methionine, the first appearance of newly synthesized albumin in the extracellular pool was about 40 min (Fig. 7 *a*). For better quantitation, the bands were digitized to yield integrated density. A plot of integrated density versus pulse/chase time confirmed the above qualitative result (Fig. 7 *b*). The initial accumulation of intracellular albumin is linear, suggesting no rapid degradation. The transit time, defined as the lag time between the linear portions of the intracellular and ex-

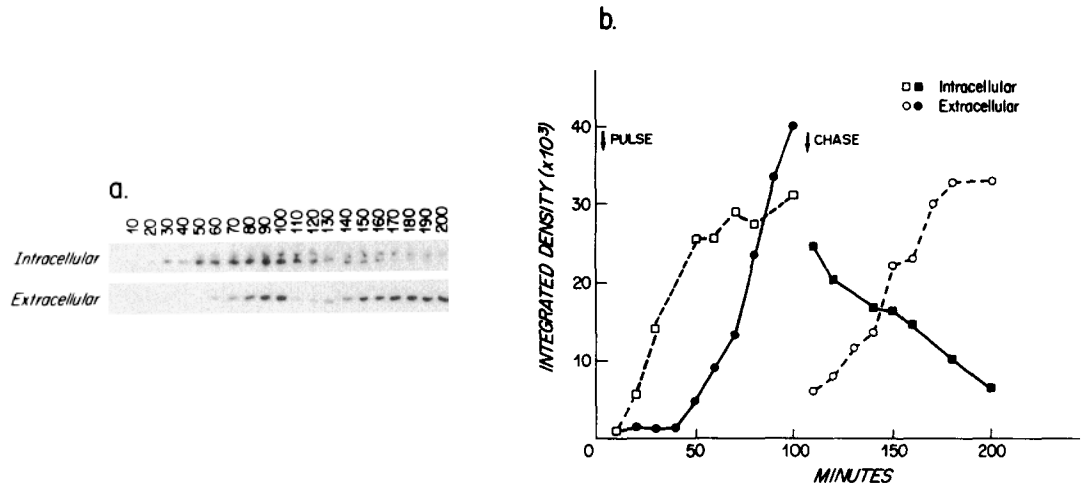

Figure 7. Secretion kinetics of albumin for cells cultured for one day as measured by pulse-chase experiments. (*a*) Autoradiograms of SDS-PAGE of immunoprecipitated intracellular and extracellular pools of albumin. Cultured cells were labeled for 100 min with [^{35}S]methionine followed by 100 min of chase without the tracer. After 10 minutes of labeling, albumin first became detectable in the intracellular pool and eventually became saturated. After 40 min of labeling, albumin began to accumulate in the extracellular pool. After the chase, albumin decreased continuously in the intracellular pool. In the extracellular pool, albumin immediately appeared and eventually reached a plateau. Repeated experiments using different batches of cells yielded similar results. (*b*) The autoradiograms were quantified by digitization. The initial accumulation of intracellular labeled albumin appears to be linear. The lag time between the linear portions of intracellular and extracellular pools during the pulse was ~40 min. After 100 min of chase, the amount of intracellular albumin present prior to the chase was completely recovered in the extracellular pool, leaving minor amount of residual albumin.

tracellular accumulation of labeled albumin, was roughly 40 min. After 100 min of labeling, culture medium was replaced to commence chasing. All of the intracellular labeled albumin before the chase were found in the extracellular pool afterwards. Therefore, synthesized albumin was quantitatively secreted.

Discussion

From the data presented above, it appears that the sandwich system differed from the single gel system primarily in the albumin gene transcriptional activity, which resulted in different levels of cellular albumin mRNA. The level of albumin mRNA, however, was not the sole determinant of albumin production rate. For both the sandwich and the single gel systems, the translational activity gradually increased with cultured time. Posttranslationally albumin appeared to be readily secreted quantitatively.

Differentiated hepatocytes contain specific mRNAs not expressed in most other cell types. From hybridization experiments, it is apparent that the albumin mRNA level in the sandwich system is similar to that observed in freshly isolated cells and is much higher than that in the single gel system. This comparison, however, is based on equal amounts of total RNA and reflects the fractional abundance of albumin mRNA. To compare the absolute amount of total albumin mRNA, one also needs to measure the total amount of RNA. The RNA content remained fairly constant in the sandwich system but decreased in the single gel system. Thus, the difference between the single gel and sandwich systems in absolute amounts of albumin mRNA was even greater than that reflected in Fig. 2 *b*. Interestingly the amount of RNA per unit of DNA increased in both systems with culture time, suggesting an increase in ribosomal RNA per cell. In addition to albumin, other liver-specific mRNAs including transferrin, ligandin, and α_1-antitrypsin were also maintained in hepatocytes cultured in the sandwich system but not in the single gel system (data not shown). Thus, hepatocytes cultured in the sandwich system appeared to mimic the in vivo pattern of mRNA expression.

The level of mRNA is determined from the balance of its synthesis and degradation. Conceivably hepatocytes cultured in the sandwich system could have maintained high levels of albumin mRNA by stabilizing the existing albumin mRNA without synthesizing new transcripts. The results from nuclear run-off analysis and rescue experiments showed that albumin gene transcription was active in the sandwich system. In rescue experiments where a second layer of collagen gel was added to the single gel system after seven days of culture, the level of albumin mRNA increased after the addition of the second layer of collagen, thereby indirectly showing active synthesis of albumin gene transcripts. The rescue experiments also demonstrated that the single gel system undergoes reversible de-differentiation, and its re-differentiation is the result of its conversion into a sandwich system. Since our initial report (15), others have also shown an increase in albumin mRNA level and albumin production when a solution of Matrigel was overlaid on hepatocytes cultured on type I collagen (6). Results from the rescue experiments showed that type I collagen overlay was as effective in the induction of the observed increase in albumin mRNA and albumin production.

Nuclear run-off assays showed that higher transcriptional activity was responsible for the higher level of albumin mRNA in hepatocytes cultured in the sandwich system compared to the single gel system. Similar results were also obtained for other liver-specific markers including transferrin, ligandin, and tyrosine aminotransferase (data not shown). The underlying assumptions involved in using this assay as a transcription indicator include: (*a*) isolated nuclei retain the in vivo transcriptional activity; (*b*) the rate of polymerase elongation is the same for different samples; and (*c*) the total rate of transcription is the same for different samples. In spite of a universal lack of testing the validity of these assumptions, this technique remains the standard for measuring transcriptional activity. Based on the correlation of transcriptional activity with mRNA level, Darnell suggested transcriptional control of normal liver gene expression (14). This is in agreement with our result, i.e., higher transcriptional activity is associated with higher level of mRNA in the sandwich system. On the other hand, as compared to the liver, the sandwich system has the same level of mRNA but only half of the transcriptional activity. This is in agreement with the literature that suggests increased mRNA stability in cultured hepatocytes (30). However, mRNA degradation kinetics needs to be directly measured to confirm this result.

Other systems in the literature capable of long-term maintenance of differentiated hepatocytes require the use of coculture, complex extracellular matrix, or special medium to achieve roughly the same level of albumin gene transcriptional activity as the sandwich system, which uses a simple matrix molecule and a simple medium. On the other hand, these other systems do not seem to require the sandwich configuration. Is there, then, a unifying theme that ties these rather disparate approaches together? One possible common denominator may be the establishment of proper cellular polarity. In the sandwich system, hepatocytes are forced to have two basolateral surfaces separated by a belt of apical surface. One may postulate that this effect is achieved in different ways by the apparently different approaches. For example, the net effect of co-culture may be to sandwich the hepatocytes between extracellular matrix proteins produced by the additional cell line, and the net effect of complex extracellular matrix is to induce the cells to become clustered so that the proper cellular polarity is still maintained.

The phenomenon of extracellular matrix modulated gene expression is frequently observed in biological systems, although no clear mechanism has been elucidated. It is interesting to speculate on the mechanism by which higher transcriptional activity is maintained in the sandwich system. Since it differs from the single gel system only in the additional layer of collagen gel, the configuration of the extracellular matrix appears to be an important factor. We have previously shown that the actin filaments are changed from the formation of abnormal stress fibers to organized subplasma membrane distribution when hepatocytes are sandwiched between collagen gels (16). This suggests that cytoskeletal filaments may mediate necessary signals to the nucleus that result in liver-specific gene transcription. However, other mechanisms such as the induction of specific extracellular matrix or surface receptor expression can not be ruled out.

In addition to transcriptional control, translational control also appeared to influence the eventual rate of albumin

production. Polyribosome size assay reflects translational activity as long as the rate of polypeptide elongation is the same for different samples. This is a common assumption made by investigators who study translational control in different systems (18, 22, 52). The data suggest that immediately after isolation hepatocytes translate albumin mRNA inefficiently. With culture time, the size of polyribosome associated with albumin mRNA gradually increases until it is tripled at the end of two weeks. This is presumably a result of damage inflicted on hepatocytes during the process of cell isolation which is reversed slowly in both the sandwich and the single gel systems. This effect is not specific for albumin; the total polyribosome profile becomes shifted towards small polyribosomes and gradually shifts back with culture time (data not shown). The overall effect on the sandwich system is a gradual increase in albumin production. For the single gel system, because of the more rapid decline in albumin mRNA content, albumin production rate is not increased. This also explains the steep increase in albumin production observed in the rescue experiments (15). Presumably the newly synthesized albumin mRNAs were translated more actively in the rescued cells compared to those in hepatocytes after one day of culture.

The secretion kinetics of synthesized albumin was assessed with pulse-chase experiments. This was performed with hepatocytes cultured on polystyrene dishes because of the large dead volume associated with collagen gel that complicates the experimental design. Because hepatocytes cultured on polystyrene ceased albumin production after a week similar to the single gel system, the available data do not cover a wide range of culture time. Nevertheless, the experiment from the first day of culture is critical to disprove the hypothesis that the initial low rate of albumin production is the result of internal degradation. It appears that all synthesized albumin are secreted, and the transit time of secretion is roughly the same as other reported values (1, 43).

In summary, the expression of liver-specific function in cultured hepatocytes was shown to be dependent on the configuration of extracellular matrix. Hepatocytes in the sandwich system maintained higher albumin mRNA content than those in the single gel system. This appeared to be the result of higher albumin gene transcriptional activity in the sandwich system. The rate of albumin production was additionally modulated by translational control, which was not correlated with the culture configuration. An increase in translational activity over time was observed for both the single gel and the sandwich systems. The secretory mechanism of albumin was intact in cultured hepatocytes. A normal transit time and quantitative secretion of all synthesized albumin were observed. A synthesis of similar features present in long-term culture systems in the literature may be the key to establish the necessary and sufficient conditions for optimal maintenance of hepatocytes.

Appendix

Calculation of Polyribosome Size

While it is possible to separate polyribosomes of different sizes on a sucrose gradient, the absorbance profile obtained from the gradient does not have sufficient resolution to distinguish polyribosomes with more than six ribosomes under the given experimental condition. Consequently, one needs to estimate the sizes of the polyribosomes in the region where distinct peaks are not resolved based on available measurements and theoretical considerations.

In the ultracentrifuge at steady state, the forces of centrifugation, buoyancy, and friction are balanced such that particles travel at velocity which is proportional to w^2r, where w is the angular speed and r is the distance from axis of rotation. The proportionality constant is called the sedimentation coefficient, s. Mathematically, the motion of the particle can be described by the following differential equation (5):

$$\frac{dr}{dt} = s\omega^2 r \qquad (1)$$

at $t = 0$, $r = r_0$

r = distance from the axis of centrifugation
r_0 = distance to the top of the gradient
t = time of centrifugation

For the purpose of comparison to other experimental settings, s is converted to a standard sedimentation coefficient, $s_{20,w}$, which refers to sedimentation in water at 20°C, by the following equation (5):

$$\frac{s_{20,w}}{s} = \frac{D_p - D_{20,w}}{D_p - D_m}\frac{\mu_m}{\mu_{20,w}} \qquad (2)$$

D_p = density of the particle in medium
$D_{20,w}$ = density of the particle in water at 20°C
D_m = density of the medium
μ_m = viscosity of the medium
$\mu_{20,w}$ = viscosity of water at 20°C

Substituting Eq. 2 into Eq. 1, one obtains the following.

$$s_{20,w}\,\omega^2\,dt = \frac{D_p - D_{20,w}}{D_p - D_m}\frac{\mu_m}{\mu_{20,w}}\frac{1}{r}\,dr \qquad (3)$$

Since the density and viscosity of the medium changes continuously in the sucrose gradient (as a function of r), this equation needs to be solved numerically. Alternatively one can use the available data to generate a calibration

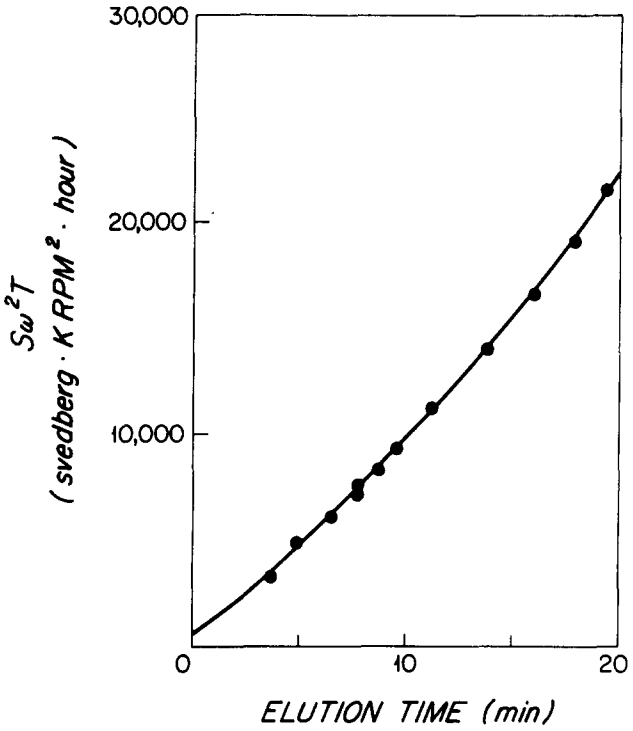

Figure 1a. Calibration curve for the estimation of large-size polyribosome was generated by the method outlined in the Appendix. $S_{20,w}\omega^2T$ was plotted against the elution time of the corresponding peak. The data points were fitted to a quadratic equation.

curve. Integration of Eq. 3 yields $F(r) = s_{20,w}\, w^2T$, where T is the total centrifugation time. For a given experiment, w^2T is constant. Therefore, F(r) is essentially a calibration curve that relates the sedimentation coefficient to distance of migration.

From the absorbance profile one can estimate the elution times of the first six sizes of polyribosomes. Elution time is linearly related to r if one approximates the gradient as a cylinder. The size of the polyribosome is related to the sedimentation coefficient by long $s_n = log\ S_1 + 0.6\ log\ n$, where n is the number of ribosomes, and S_1 is the standard sedimentation coefficient of monoribosome, 83 (42). By plotting $s_{20,w}\, w^2T$ versus corresponding elution time, one generates the desired calibration curve. However, as noted earlier, available data do not cover the entire range of elution time. This can be remedied by performing prolonged ultracentrifugation such that the smaller polyribosomes sediment closer to the bottom of the tube, thereby increasing the quantity $s_{20,w}\, w^2T$. Fig. 5 shows the polyribosomes profiles from two centrifugations runs for 1.5 and 3.5 h at 26,000 rev/min in an SW28 rotor. One can readily see the locations of the first six polyribosomal peaks, which correspond to the elution times of monoribosome, diribosome, etc. The sizes of polyribosome are converted to their respective sedimentation values and multiplied by total centrifugation time and the square of centrifugation speed. These values are plotted versus the elution times in Fig. 1 (appendix). By fitting a quadratic equation to the data, one obtains a relation that gives the sedimentation coefficient as a function of elution time. The sedimentation coefficient can be converted back to the corresponding size of polyribosome. This new function can be averaged over the periods of fractionation, i.e., 0 to 3 min, 3 to 6 min, etc., yielding the mean polyribosome sizes for all aliquots. To obtain the average polyribosome size for a sample, one first normalizes the amount of albumin mRNA in the different aliquots. The sum of the products of the mean polyribosome size and the relative abundance of albumin mRNA in each aliquot gives the polyribosome size for the sample (20).

We thank Dr. Harvey Lodish for his helpful discussion and Michael D. Harmon, Lauren H. Kim, Eve J. Smith, and Laura M. Sterling for their technical assistance.

This work was supported by National Institute of Diabetes and Digestive and Kidney Disease CIA DK-01746 and RO1 DK-41709 and a grant from Shriner's Burn Institute. M. L. Yarmush is a Lucille P. Markey fellow in biomedical science, J. C. Y. Dunn is a National Science Foundation fellow.

Received for publication 24 June 1991 and in revised form 30 October 1991.

References

1. Bartles, J. R., H. M. Feracci, B. Stieger, and A. L. Hubbard. 1987. Biogenesis of the rat hepatocytes plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241–1251.
2. Ben-Ze'ev, A., G. S. Robinson, N. L. Bucher, and S. R. Farmer. 1988. Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary culture of rat hepatocytes. *Proc. Natl. Acad. Sci. USA.* 85:2161–2165.
3. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* 43:506–520.
4. Bissell, D. M., D. M. Arenson, J. J. Maher, and F. J. Roll. 1987. Support of cultured hepatocytes on a laminin-rich gel. *J. Clin. Invest.* 79:801–812.
5. Cantor, C. R., and P. R. Schimmel. 1980. Velocity sedimentation. *In* Biophysical Chemistry Part II: Techniques for the Study of Biological Structure and Function. W. H. Freeman and Company, San Francisco, CA. 591–604.
6. Caron, J. M. 1990. Induction of albumin gene transcription in hepatocytes by extracellular matrix proteins. *Mol. Cell. Biol.* 10:1239–1243.
7. Carter, E. A., and J. R. Wands. 1988. Ethanol-induced inhibition of liver cell function: I. Effect of ethanol on hormone stimulated hepatocyte DNA synthesis and the role of ethanol metabolism. *Alcohol. Clin. Exp. Res.* 12:555–562.
8. Cathala, G., J. F. Savouret, B. Mendez, B. L. West, M. Karin, J. A. Martial, and J. D. Baxter. 1983. A method for isolation of intact, translationally active ribonucleic acid. *DNA.* 2:329–335.
9. Chambard, M., J. Gabrion, and J. Mauchamp. 1982. Influence of collagen gel on the orientation of epithelial cell polarity: follicle formation from isolated thyroid cells and from preformed monolayers. *J. Cell Biol.* 91: 157–166.
10. Chen, J., E. C. Stuckey, and C. L. Berry. 1985. Three-dimensional culture of rat exocrine pancreatic cells using collagen gels. *Br. J. Exp. Pathol.* 66:551–559.
11. Clayton, D. F., and J. E. Darnell. 1983. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. *Mol. Cell. Biol.* 3:1552–1561.
12. Clayton, D. F., A. L. Harrelson, and J. E. Darnell. 1985. Dependence of liver-specific transcription on tissue organization. *Mol. Cell. Biol.* 5: 2623–2632.
13. Demetriou, A. A., A. Reisner, J. Sanchez, S. M. Levenson, A. D. Moscioni, and J. R. Chowdhury. 1988. Transplantation of microcarrier-attached hepatocytes into 90% partially hepatectomized rats. *Hepatology.* 8:1006–1009.
14. Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell. 1981. Transcriptional control in the production of liver-specific mRNAs. *Cell.* 23:731–739.
15. Dunn, J. C. Y., M. L. Yarmush, H. G. Koebe, and R. G. Tompkins. 1989. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3: 174–177.
16. Dunn, J. C. Y., R. G. Tompkins, and M. L. Yarmush. 1991. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnology Progress.* 7:237–245.
17. Elsdale, T., and J. Bard. 1972. Collagen substrata for studies on cell behavior. *J. Cell Biol.* 54:626–637.
18. Fagan, R. J., A. Lazaris-Karatzas, N. Sonenberg, and R. Roze. 1991. Translational control of ornithine aminotransferase: modulation by initiation factor eIF-4E. *J. Biol. Chem.* 266:16518–16523.
19. Fausto, N. 1986. New perspective on liver regeneration. *Hepatology.* 6:326–327.
20. Fioretti, W. C., D. F. Davis, and B. E. Ledford. 1979. Polyribosomes size analysis: measurement of number-average polyribosome sizes. *Biochem. Biophys. Acta.* 564:79–89.
21. Fraslin, J. M., B. Kneip, S. Vaulont, D. Glase, A. Munnich, and C. Guguen-Guillouzo. 1985. Dependence of hepatocyte-specific gene expression on cell-cell interactions in primary culture. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2487–2491.
22. Garrett, J. E., M. W. Collard, and J. O. Douglass. 1989. Translational control of germ cell-expressed mRNA imposed by alternative splicing: opioid peptide gene expression in rat testis. *Mol. Cell Biol.* 9:4381–4389.
23. Guguen-Guillouzo, C., B. Clement, G. Baffet, C. Beaumont, E. Morel-Chany, D. Glaise, and A. Guillouzo. 1983. Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. *Exp. Cell. Res.* 173:47–54.
24. Hall, H. G., D. A. Farson, and M. J. Bissell. 1982. Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. *Proc. Natl. Acad. Sci. USA.* 79:4672–4676.
25. Hay, E. D. 1981. Extracellular matrix. *J. Cell Biol.* 91:205s–223s.
26. Ingber, D. E., J. A. Madri, and J. D. Jamieson. 1986. Basement membrane as a spatial organizer of polarized epithelia. *Am. J. Pathol.* 122:129–139.
27. Isom, H. C., T. Secott, I. Georgoff, C. Woodworth, and J. Mummaw. 1985. Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci. USA.* 82:3252–3256.
28. Isom, H., I. Georgoff, M. Salditt-Georgieff, and J. E. Darnell. 1987. Persistence of liver-specific messenger RNA in cultured hepatocytes: different regulatory events for different genes. *J. Cell Biol.* 105:2877–2885.
29. Jeejeebhoy, K. N., A. Bruce-Robertson, J. Ho, and U. Sodtke. 1973. Protein turnover. *Ciba Found. Symp.* 9:217–238.
30. Jefferson, D. M., D. F. Clayton, J. E. Darnell, and L. M. Reid. 1984. Post-transcriptional modulation of gene expression in cultured rat hepatocytes. *Mol. Cell. Biol.* 4:1929–1934.
31. Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473–485.
32. Koebe, H. G., J. C. Y. Dunn, M. Toner, L. M. Sterling, A. Hubel, E. G. Cravalho, M. L. Yarmush, and R. G. Tompkins. 1990. A new approach to the cryopreservation of hepatocytes in a sandwich culture configuration. *Cryobiology.* 27:576–584.
33. Kreamer, B. L., J. L. Staecker, N. Sawada, G. L. Sattler, M. T. Hsia, and H. Pitot. 1986. Use of a low-speed, iso-density percoll centrifugation method to increase viability of isolated rat hepatocytes preparations. *In Vitro Cell. Dev. Biol.* 22:201–211.
34. Lee, E. Y. P., W. Lee, C. S. Kaetzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. *Proc. Natl. Acad. Sci. USA.* 82:1419–1423.
35. Leffert, H. L., and D. Paul. 1972. Studies on primary cultures of differentiated fetal liver cells. *J. Cell Biol.* 52:559–568.
36. Lodish, H. F., and S. Froshauer. 1977. Rates of initiation of protein synthesis by two purified species of vesicular stomatitis virus messenger RNA. *J. Biol. Chem.* 252:8804–8811.
37. Maher, J. J. 1988. Primary hepatocyte culture: is it home away from home? *Hepatology.* 8:1162–1166.
38. Martin, G. R., and R. Timpl. 1987. Laminin and other basement membrane components. *Annu. Rev. Cell Biol.* 3:57–85.
39. Mesnil, M., J. M. Fraslin, C. Piccoli, H. Yamasaki, and C. Guguen-Guillouzo. 1987. Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp. Cell Res.* 173:524–533.

40. Michalopoulos, G., and H. C. Pitot. 1975. Primary culture of parenchymal liver cells on collagen membranes. *Exp. Cell Res.* 94:70–78.
41. Montesano, R., P. Mouron, M. Amherdt, and L. Orci. 1983. Collagen matrix promotes reorganization of pancreatic endocrine cell monolayers into islet-like organoids. *J. Cell Biol.* 97:935–939.
42. Noll, H. 1969. Polysomes: Analysis of Structure and Function. Techniques in Protein Biosynthesis. Campbell, P.N., and J. R. Sargent, editors. Academic Press, New York. Vol. II: 101–179.
43. Peters, T. 1985. Serum albumin. *Adv. Protein Chem.* 37:161–215.
44. Powell, D. J., J. M. Friedman, A. J. Oulette, K. S. Krauter, and J. E. Darnell. 1984. Transcriptional and post-transcriptional control of specific messenger RNAs in adult and embryonic liver. *J. Mol. Biol.* 179:21–35.
45. Reid, L. M., and D. M. Jefferson. 1984. Culturing hepatocytes and other differentiated cells. *Hepatology.* 4:548–559.
46. Reid, L. M., Z. Gaitmaitain, I. Arias, P. Ponce, and M. Rojkind. 1980. Long-term cultures of normal rat hepatocytes on liver biomatrix. *Ann. NY Acad. Sci.* 349:70–76.
47. Schuetz, E. G., D. Li, C. J. Omiecinski, U. Muller-Eberhard, H. K. Kleinman, B. Elswick, and P. S. Guzelian. 1988. Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *J. Cell Physiol.* 134:309–323.
48. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Biol.* 13:29–83.
49. Spray, D. C., M. Fujita, J. C. Saez, H. Choi, T. Watanabe, E. Hertzberg, L. C. Rosenberg, and L. M. Reid. 1987. Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. *J. Cell Biol.* 105:541–551.
50. Tompson, J. A., C. C. Haudenschild, K. D. Anderson, J. M. DiPietro, W. F. Anderson, and T. Maciag. 1989. Heparin-binding growth factor 1 induces the formation of organoid neovascular structures *in vivo*. *Proc. Natl. Acad. Sci. USA.* 86:7928–7932.
51. Uchino, J., T. Tsuburaya, F. Kumagai, T. Hase, T. Hamada, T. Komai, A. Funatsu, E. Hashimura, K. Nakamura, and T. Kon. 1988. A hybrid bioartificial liver composed of multiplated hepatocyte monolayers. *Trans. Am. Soc. Artif. Intern. Organs.* 34:972–977.
52. Weinberg, A. D., and S. L. Swain. 1990. IL-2 receptor (Tac antigen) protein expression is down-regulated by the 5′-untranslated region of the mRNA. *J. Immunol.* 144:4712–4720.
53. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.
54. Yang, J., D. Flynn, L. Larson, and S. Hamamoto. 1982. Growth in primary culture of mouse submandibular epithelial cells embedded in collagen gels. *In Vitro.* 18:435–442.