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Recovery of mRNA Expression of Tryptophan 2,3-Dioxygenase and Serine Dehydratase in Long-Term Cultures of Primary Rat Hepatocytes[1]

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Expression of tryptophan 2,3-dioxygenase (TO) and serine dehydratase (SDH) has not previously been maintained or re-induced in long-term cultured hepatocytes. In the present study, we succeeded in inducing expression of TO and SDH mRNAs in adult rat hepatocytes cultured in serum-free L-15 medium supplemented with epidermal growth factor and 2% dimethyl sulfoxide (DMSO). After the start of culture, the expression of TO mRNA rapidly disappeared and at 96 h it was less than 10% of that at isolation. However, after the addition of 2% DMSO from 96 h, the transcript level gradually increased and reached about 40% of that of the isolated cells at day 14. In addition, the expression of TO mRNA was enhanced in cells treated with both 10^{-5} M dexamethasone and 10^{-7} M glucagon. In contrast, the expression of SDH mRNA decreased very rapidly and we could not detect it after 24 h of culture. Furthermore, 2% DMSO failed to induce it. However, when both 10^{-5} M dexamethasone and 10^{-7} M glucagon were added to the culture medium at day 9, we observed dramatic induction of SDH mRNA 24 h later. Primary hepatocytes cultured by this method could express and maintain highly differentiated hepatic functions for a long time. Thus, this *in vitro* system is suitable for the investigation of hepatic functions.

Key words: dimethyl sulfoxide, primary rat hepatocytes, re-expression, serine dehydratase, tryptophan 2,3-dioxygenase.

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 environmental changes (*1*). Therefore, parenchymal hepatocytes represent an excellent system to study the regulation of gene expression, as well as its relation to cellular differentiation and growth control. To reproduce hepatic functions *in vitro* similar to those *in vivo*, many researchers have been using primary cultured hepatocytes (*2-11*). However, it has been very difficult to maintain the differentiated functions of the hepatocytes, especially the levels of tryptophan 2,3-dioxygenase (EC 1.13.11.11; TO) (*10, 12, 13*) and serine dehydratase (EC 4.2.1.13; SDH) (*14, 15*). Neither enzyme has been reported to be expressed by any established liver cell line derived from either normal liver or well-differentiated hepatoma cells (*16, 17*). In addition, the expression of mRNAs of both enzymes rapidly disappears from primary cells. Until now, the re-induction and maintenance of these enzymes have never been reported using primary cultures of rat hepatocytes.

Dimethyl sulfoxide (DMSO) is well known to induce differentiation in some cell lines (*18-20*). For the purpose of maintaining differentiated hepatic functions, 2% DMSO is also used in primary cultures of rat hepatocytes (*21-24*). Recently, we established a culture system in which, by adding 2% DMSO to the culture medium after the hepatocytes proliferated, the cells were able to recover differentiated functions such as albumin and transferrin secretion and glucose-6-phosphatase activity (*25*). In addition, gap-junctional proteins, connexin 32 (Cx 32) and 26 (Cx 26), could reappear and be maintained, together with extensive gap-junctional intercellular communication for more than one month (*26, 27*). In the present experiment, we examined the re-expression and maintenance of mRNAs of TO and SDH in primary rat hepatocytes cultured in modified L-15 medium supplemented with epidermal growth factor (EGF) and 2% DMSO.

MATERIALS AND METHODS

Isolation and Culture of Rat Hepatocytes—Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center,

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Abbreviations: TO, tryptophan 2,3-dioxygenase; SDH, serine dehydratase; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; Cx, connexin; L.I., labeling index; ELISA, enzyme-linked immunosorbent assay; BrdU, 5-bromo-2'-deoxyuridine; TAT, tyrosine aminotransferase.

Hamamatsu), weighing about 300-400 g, were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (*28*) with some modifications (*25*). Briefly, the liver was perfused *in situ* through the portal vein with 150 ml of Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (Sigma Chemical, St. Louis, USA), 0.5 mg/liter insulin (Sigma Chemical) and antibiotics. After the initial perfusion, the liver was perfused with 200 ml of HBSS containing 40 mg of collagenase (Yakult, Tokyo) for 10 min. The isolated cells were purified by Percoll iso-density centrifugation (*29*). Viability of the cells, as evaluated by the trypan blue exclusion test, was more than 90% in these experiments. The cells were suspended in L-15 medium with 0.2% bovine serum albumin (BSA; Seikagaku Kogyo, Tokyo), 20 mM HEPES (Dojindo, Kumamoto), 0.5 mg/liter insulin (Sigma Chemical), 10^{-7} M dexamethasone (Sigma Chemical), 1 g/liter galactose, 30 mg/liter proline, and antibiotics. The hepatocytes were plated on 35- or 60-mm culture dishes (Corning Glass Works, Corning, USA), which were coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid) (*30*), and placed in a 100% air incubator at 37°C. Two to 3 h after plating, the medium was changed to L-15 medium supplemented with 0.2% BSA, 20 mM HEPES, 0.5 mg/liter insulin, 10^{-7} M dexamethasone, 1 g/liter galactose, 30 mg/liter proline, 20 mM $NaHCO_3$, 5 mg/liter transferrin (Wako Pure Chemical, Osaka), 0.2 mg/liter $CuSO_4 \cdot 5H_2O$, 0.5 mg/liter $FeSO_4 \cdot 4H_2O$, 0.75 mg/liter $ZnSO_4 \cdot 7H_2O$, 0.05 mg/liter $MnSO_4$, 5 μg/liter Na_2SeO_3, 10 μg/liter EGF (Collaborative Res., Lexington, USA), and antibiotics. The cells were then placed in a humidified, 5% CO_2:95% air incubator at 37°C. The medium was replaced with fresh medium every other day, and 2% DMSO (Aldrich Chemical, Milwaukee, USA) was added from day 4 (96 h after plating).

Labeling Index—To examine the labeling indices (L.I.), immunocytochemical staining for 5-bromo-2′-deoxyuridine (BrdU) was carried out. First, 20 μM BrdU was added to each 35-mm dish 24 h before fixation and the cells were fixed in cold absolute ethanol. Mouse anti-BrdU antibody (DAKOPATTS A/S, Denmark) was used as the primary antibody, followed by the ABC method (Vectastain ABC Elite kit, Vector Laboratory, Burlingame, USA). Labeled cells that had nuclei stained for BrdU were counted by the use of a microscope (magnification×200). Twenty fields per dish were observed. More than 1,000 cells were counted per dish and three dishes were examined per experiment. For statistical analysis, *t* tests were performed.

ELISA for Rat Albumin—Quantification of the secreted rat albumin was done by enzyme-linked immunosorbent assay (ELISA) (*31*). We used the two-antibody sandwich method. The medium of two dishes was collected every 48 h and centrifuged at 10,000 rpm for a few seconds. The supernatant was transferred to a new Eppendorf tube and kept at −20°C until use. Goat anti-rat albumin antibody (0.5 μg/well; Cappel, West Chester, USA) was bound to each well of a 96-well microtiter plate (Dynatech Lab., Chantilly, USA). After incubation with blocking buffer (70% Block Ace [Dainippon Pharm, Osaka]/0.05 M PBS) overnight, samples (1.2×10^4 times dilution of the culture medium) were applied to the wells. Rat albumin (Sigma Chem.) was used as a standard. Then, 0.5 μg of peroxidase-conjugated rabbit anti-rat albumin antibody (Cappel) was added to each well. ABTS (Boehringer Mannheim Biochemica, Germany) was used as a chromogenic substrate. A microplate reader (Dynatech Lab., MR600) was used for the measurement.

Northern blot analysis and densitometry: Total RNA was extracted from the cells using the single-step thiocyanate-phenol-chloroform extraction method (*32*) as modified by Xie and Rothblum (*33*). For electrophoresis, 10 or 30 μg of total RNAs was loaded on 1% agarose gel containing 0.5 mg/liter ethidium bromide. Gels were capillary-blotted in 20×standard saline citrate (SSC) onto a nylon membrane (Hybond-N; Amersham, Buckinghamshire, UK) and fixed by exposure to ultraviolet light.

For the detection of albumin, TO and SDH mRNAs, membranes were prehybridized in a solution containing 50% formamide, 0.9 M NaCl, 0.1 M $NaPO_4$ (pH 7.4), 1% SDS, 10 mg/liter herring sperm DNA, and 5×Denhart's solution for 4 h at 42°C, and then hybridized overnight at 42°C in the same solution with a ^{32}P-labeled cDNA probe. The cDNA probe of rat albumin (partial 1.0 kb *Pst*I fragment from the 3′-end; a generous gift from M. Sakai), TO (full 1.7 kb *Eco*RI fragment) (*34*) and SDH (full 1.45 kb *Eco*RI fragment; from R. Kanamoto) (*35*) were used. Thereafter, the membranes were washed twice in 2×SSC buffer containing 0.1% SDS for 5 min at RT and twice in 2× SSC buffer containing 1% SDS for 30 min at 68°C before exposure to X-ray film. Scanning densitometry was performed using a Macintosh Quadra 800 computer (Apple Computer, Cupertino, USA) and an EPSON GT-6000 scanner (Seiko Epson, Suwa). The signals were quantified by the NIH Image 1.52 Densitometric Analysis Program (Wayne Rasband, NIH, Bethesda, USA) (*36*).

RESULTS

Primary rat hepatocytes were cultured in modified L-15 medium with 10 μg/liter EGF and 20 mM $NaHCO_3$ in a 5% CO_2:95% air incubator and maintained in medium containing 2% DMSO from day 4. The cells exhibited very few divisions until day 2, but many mitoses were observed after day 3, as previously reported (*25*). Two-percent DMSO has been used for the maintenance of hepatic differentiated functions (*4, 22*) and for the inhibition of DNA synthesis of primary rat hepatocytes (*21, 23, 24*). In the present experiment we examined the inhibitory effect of 2% DMSO on the DNA synthesis of the cells. Table I shows the L.I. of hepatocytes treated with or without 2% DMSO. In the period from 24-48 h about 20% of the cells were labeled, but in the following 24 h period the L.I. remarkably increased to about 60%. Following this, the L.I. gradually decreased with time in culture. An inhibitory effect of 2% DMSO on the DNA synthesis was seen in the period from 96-120 h. More than 50% inhibition was observed in the cells treated with 2% DMSO. Thereafter, only a small percentage of the cells was labeled in the cultures with 2% DMSO.

To examine whether the hepatocytes with 2% DMSO could increase the secretion of albumin into the medium, ELISA for rat albumin was conducted. Until day 4, the proliferating hepatocytes secreted about 200 mg/liter/48 h of albumin into the medium (Fig. 1). Thereafter, the secretion of albumin decreased with time in culture when

the cells were cultured without DMSO. However, after the addition of 2% DMSO, the secretion gradually increased and reached about 500 mg/liter/48 h in the period from days 12-14.

Figure 2A shows the changes in transcript expression of albumin and TO mRNA in hepatocytes with (+) or without (−) 2% DMSO. Figure 2B shows the percentage change of the mRNA expression. The transcription of mRNAs in the isolated cells was 100%. The expression of albumin mRNA gradually decreased with time in culture. The expression at 96 h after plating was about one-half that at the isolation and decreased to less than 10% at day 14. However, when 2% DMSO was added to the medium at 96 h, the expression of albumin mRNA gradually increased and recovered to two-thirds that at the time of isolation by day 14. On the other hand, the expression of TO mRNA rapidly decreased after 24 h although a slight increase was observed at 8 h after plating. The transcription at 96 h was less than 10% of that at isolation and after day 8 the expression was no longer detectable. The hepatocytes treated with 2% DMSO gradually recovered TO mRNA expression and the level reached about 40% of that of the isolated cells by day 14. The TO mRNA expression was maintained for more than 2 wk (data not shown).

TABLE I. **Labeling indices of cultured hepatocytes (%).**

Hours after plating	Control	2% DMSO[a]
24-48	23.7±5.9[b]	
48-72	59.1±6.9	
72-96	41.0±5.8	
96-120	24.8±1.9	9.4±0.2*
120-144	18.5±2.7	1.4±0.1*
144-168	15.8±2.4	3.3±0.1*
168-192	10.6±0.6	4.6±1.7*
192-216	10.9±0.7	2.7±0.6*

[a]2% DMSO was added to the culture medium at 96 h after plating. [b]These numbers are the mean±standard deviation of three dishes. The cells were treated with 20 μM BrdU for 24 h. *The value is significantly different from the control; $p<0.01$.

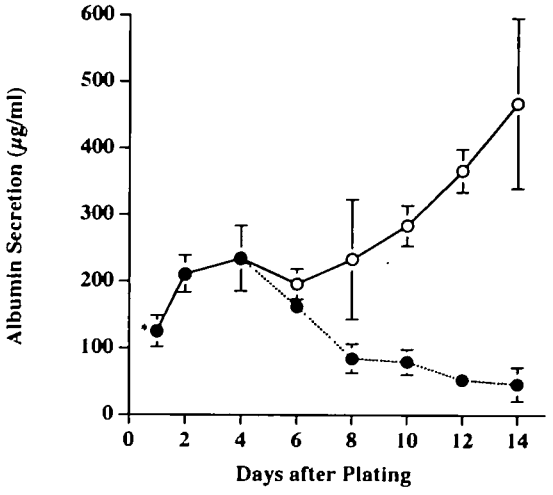

Fig. 1. **Albumin secretion into the medium in 48 h periods.** Primary rat hepatocytes cultured in modified L-15 medium with 10 μg/liter EGF (●). DMSO (2%) was added to the medium from day 4 (○). Asterisk (*) shows albumin secretion into the medium for 24 h from the time of the first medium change.

It has been reported that TO expression is regulated by hormones such as dexamethasone, glucagon and insulin (*6, 13, 37*). Therefore, we investigated the effects of those hormones on hepatocytes cultured in the medium with 2% DMSO. Figure 3A showed the changes in transcription of TO mRNA of hepatocytes with 2% DMSO from 96 h after plating, and 24 h treatment with both 10^{-5} M dexamethasone and 10^{-7} M glucagon at day 9 was carried out for the induction of TO mRNA. The transcription of TO mRNA was remarkably enhanced by the addition of both hormones and the level of expression reached about 85% of that of the

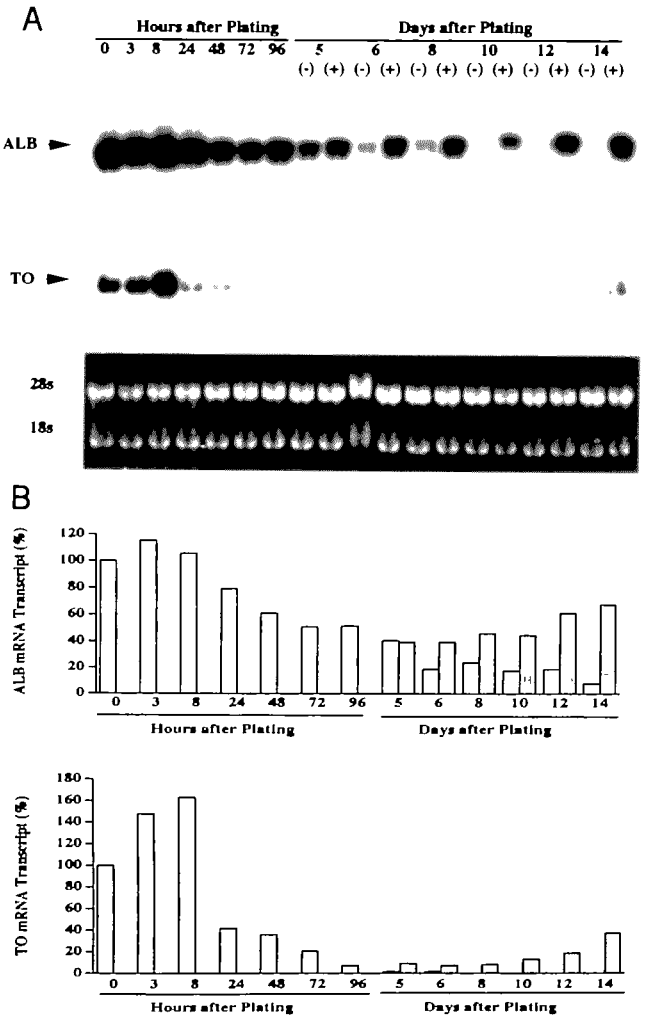

Fig. 2. **(A) Northern blot analysis for albumin and TO mRNAs of primary rat hepatocytes cultured in modified L-15 medium with 10 μg/liter EGF.** The cells were treated with (+) or without (−) 2% DMSO from 96 h. The left half shows the samples from 0 h to 96 h without 2% DMSO. The right half shows the samples from day 5 to day 14 after addition of 2% DMSO from day 4. Total RNA (10 μg/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with ^{32}P-labeled albumin and TO cDNA probes. Ethidium bromide staining of ribosomal RNAs was done before transfer to membranes. **(B) Scanning-densitometric analysis of the mRNA level was performed.** The expression of each mRNA is shown as the expression in 28 s. Expression of mRNAs of the cells with (▩) or without (□) 2% DMSO is shown as % of the 0 h value.

isolated cells by day 10 (Fig. 3B). Then we divided the cells into four groups from 96 h (Fig. 4). Group **a** used basal medium containing 10^{-7} M dexamethasone (control). Group **b** was maintained in medium containing 10^{-7} M dexamethasone and 10^{-7} M glucagon, and group **c** in medium containing 10^{-5} M dexamethasone and group **d** in medium containing 10^{-5} M dexamethasone and 10^{-7} M glucagon. Figure 4A shows the changes in transcription of albumin and TO mRNAs. Figure 4B shows the intensity measurements of the bands from Fig. 4A. At one and two days after 2% DMSO addition (day 5 and day 6), the transcription of albumin mRNA was not changed by the addition of 10^{-5} M dexamethasone and/or 10^{-7} M glucagon. The effect of both hormones on the expression appeared in the cells of day 10 and day 14. At day 14 the expression of albumin mRNA induced by both 10^{-5} M dexamethasone and 10^{-7} M glucagon increased to 1.8 times that of cells cultured in the control medium (group **a**). On the other hand, although TO mRNA expression was not influenced by the addition of 10^{-5} M dexamethasone and/or 10^{-7} M glucagon to the cells of day 5 and day 6, induction by both hormones was observed thereafter. At day 14, the TO mRNA expression of the cells of group **d** was 2.1 times that of the control. We found that the hepatocytes treated with high concentrations of both dexamethasone and glucagon for a long time could recover mRNA expression of albumin and TO better than the cells cultured in the control medium.

Figure 5 shows the changes in transcription of SDH

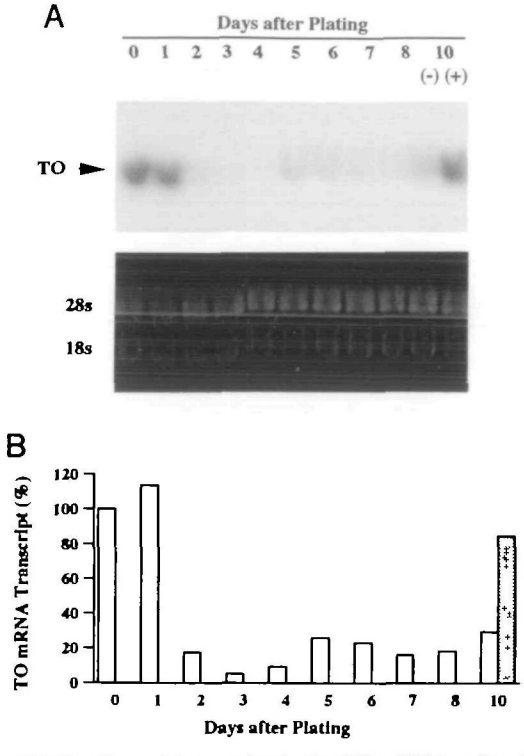

Fig. 3. **(A) Northern blot analysis for TO mRNAs of primary rat hepatocytes cultured in modified L-15 medium with 10 μg/liter EGF.** DMSO (2%) was added to the culture medium from day 4. Day 0 means the isolated hepatocytes. At day 9 the cells were treated with (+) or without (−) both 10^{-5} M dexamethasone and 10^{-7} M glucagon and, 24 h later (day 10), the cells were harvested. Total RNA (30 μg/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with a ^{32}P-labeled TO cDNA probe. Ethidium bromide staining of ribosomal RNAs was done before transfer to membranes. **(B) Scanning-densitometric analysis of the mRNA level was performed.** The expression of each mRNA is shown as the expression in 28 s. Expression of mRNAs of the cells is indicated by empty columns (□). The induction of TO mRNA by both dexamethasone and glucagon is shown by a dotted column (▨).

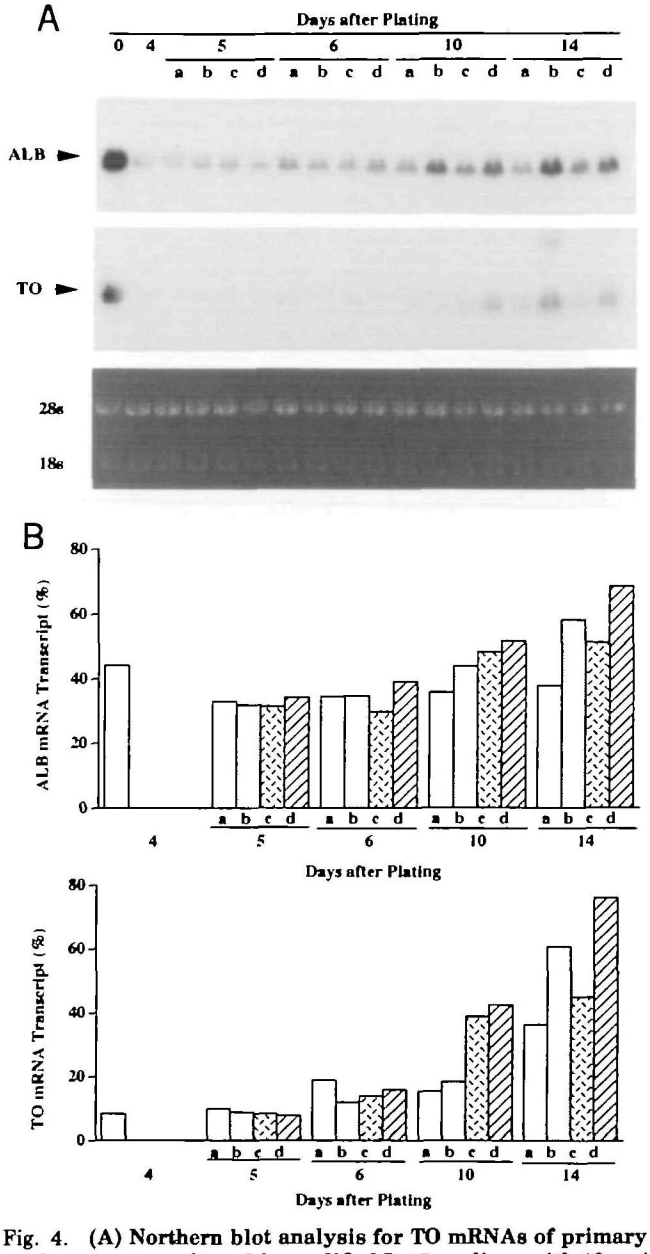

Fig. 4. **(A) Northern blot analysis for TO mRNAs of primary rat hepatocytes cultured in modified L-15 medium with 10 μg/liter EGF.** DMSO (2%) was added to the culture medium from day 4. Group **a** is the medium containing 10^{-7} M dexamethasone as basal medium (□); Group **b** is the medium containing 10^{-7} M dexamethasone and 10^{-7} M glucagon from day 4 (■); Group **c** is the medium containing 10^{-5} M dexamethasone from day 4 (▨); Group **d** is the medium containing 10^{-5} M dexamethasone and 10^{-7} M glucagon from day 4 (▨). Ethidium bromide staining of ribosomal RNAs was done before transfer to membranes. **(B) Scanning-densitometric analysis of the mRNA level was performed.** Expression of the transcripts is shown as % of the 0 h value.

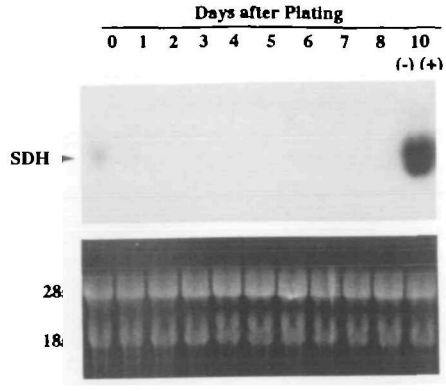

Fig. 5. **Northern blot analysis for SDH mRNAs of primary rat hepatocytes cultured in modified L-15 medium with 10 μg/liter EGF.** DMSO (2%) was added to the culture medium from day 4. Day 0 means the isolated hepatocytes. At day 9, the cells were treated with (+) or without (−) both 10^{-5} M dexamethasone and 10^{-7} M glucagon and, 24 h later (day 10), the cells were harvested. Total RNA (30 μg/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with a ^{32}P-labeled SDH cDNA probe. Ethidium bromide staining of ribosomal RNAs was done before transfer to membranes.

mRNA of hepatocytes with 2% DMSO from 96 h after plating. Although the expression of SDH mRNA was observed within 24 h after plating, it was not thereafter detected throughout the culture period, even in the cells treated with 2% DMSO. We examined whether the transcription of SDH mRNA could be induced by 10^{-5} M dexamethasone plus 10^{-7} M glucagon in the cells of day 9. Dramatic induction (6.8 times that of the isolated hepatocytes) was observed in the hepatocytes treated with both hormones for 24 h. The addition of 10^{-7} M glucagon to the culture medium was sufficient for the induction of SDH mRNA because the medium contained 10^{-7} M dexamethasone. On the other hand, increase of the concentration of dexamethasone in the medium did not induce SDH mRNA (data not shown).

DISCUSSION

Many researchers have attempted to maintain differentiated functions in primary cultured hepatocytes by using various agents such as nicotinamide (*10, 38*), phenobarbital (*5*), sodium butyrate (*9*), extracellular matrix (*3, 8*), and by forming spheroids (*11*) or by co-culturing with liver-derived epithelial cells (*39*). Recently, Dunn *et al.* (*40*) reported that adult rat hepatocytes sandwiched between two layers of collagen gel maintained a level of albumin mRNA similar to that found in the normal liver for at least six weeks. In those investigations albumin and tyrosine aminotransferase (TAT) were mainly used as markers of hepatic differentiated functions. However, expression of enzymes such as P-450, SDH, and TO, which correspond to highly differentiated functions of mature hepatocytes, has never been maintained or re-induced in primary hepatocytes cultured for a long period of time. In addition, no established cell lines and no other tissues except liver express SDH or TO (*13, 15*), although some cell lines possess albumin and/or TAT activity (*20, 41*). In the present experiment we demonstrated that proliferated rat hepatocytes could not only maintain the expression of albumin mRNA but also re-express both SDH and TO mRNAs in modified L-15 medium supplemented with EGF and 2% DMSO. Furthermore, albumin secretion by the cultured hepatocytes gradually increased and reached at least 10 mg/h/10^6 cells at day 14. This value is about 300% of the *in vivo* rate, which is estimated to be about 3 mg/h/10^6 cells (*42*). The hepatocytes cultured by this method possessed P-450 in their cytoplasm even two weeks after plating (data not shown).

TO plays a key role in the L-tryptophan metabolic pathway and SDH catalyzes degradation of serine to pyruvate and ammonia. Both enzymes are induced in gluconeogenic conditions such as starvation, diabetes, or intake of a high protein diet. In primary cultures of adult rat hepatocytes, both glucocorticoid and glucagon (cAMP) are essential for induction of SDH, whereas glucocorticoid or glucagon is necessary for induction of TO (*6*). Furthermore, insulin suppresses the induction of both enzymes by glucocorticoid plus glucagon. These multihormonal effects were reported to be regulated at the transcriptional step (*13, 43*). In the present experiment, even at day 10, the cultured hepatocytes possessed responses to the hormones similar to those described above: increase of the concentration of dexamethasone or the addition of glucagon induced TO mRNA, and SDH mRNA reappeared at a much higher level than that *in vivo* after the addition of both glucagon and dexamethasone. In addition, it is of interest that the transient induction of TO mRNA was observed at 8 h after plating. Nakamura *et al.* (*13*) reported that TO mRNA induction by dexamethasone and glucagon was highest at 12 h after the addition of the hormones. At the time of the isolation of primary hepatocytes, the cells are free from hormones for about one hour because the perfusate does not contain dexamethasone and the cells are kept on ice. Therefore, we think that the application of culture medium with dexamethasone to the isolated cells may cause hormonal induction similar to that reported by Nakamura *et al.* (*13*). On the other hand, the existence of two mRNA species of SDH was reported by Noda *et al.* (*44*) and Ogawa *et al.* (*45*). As they reported that these two mRNAs were indistinguishable by Northern blot analysis, we could not identify the species of mRNA (*46*). Further experiments are necessary to clarify whether the re-expressed SDH mRNA is mRNA I or II.

Isom *et al.* (*4*) first reported that supplementation of chemically defined medium with 2% DMSO enabled maintenance of differentiated hepatocytes in culture for extended periods. In the present and previous experiments, DMSO played an important role in the maintenance of hepatocytes (*25-27*): the agent seemed to induce the differentiated functions and structures of mature hepatocytes. DMSO is known to have a plethora of cellular effects, many of which can affect the cytoskeleton directly by activation of protein kinase C (*47*) or by changing intracellular calcium levels (*48*). These second messengers probably play a role in modulating cell shape and morphology (*49*). The DMSO-mediated effects on intracellular Ca^{2+} and/or phosphorylation levels can affect specific cell-cell or cell-matrix interactions by directly stimulating changes in second messengers associated with activation of cadherins or integrins (*49*). The mechanism by which cellular architecture is linked to differentiated function is unclear. Ben-

Ze'ev *et al.* (*50*) suggested that cell shape, which they found to be controlled by cell-cell contact and cell-matrix interactions, might be a primary regulator of tissue-specific gene expression and that cytoskeletal components might interact directly with the nuclear matrix to affect gene transcriptional rates. Singhvi *et al.* (*51*) recently reported that they concurrently regulated hepatocyte albumin secretion by controlling cell shape using an imprinted substratum. A decrease in the size of the adhesive island resulted in a progressive reduction in growth and in an increase of albumin production. In our present experiment, differing from conventional conditions, primary hepatocytes were plated on dishes at subconfluent density and could proliferate to reach about twice the initial density by day 6 (*25*). In addition, a thick coating of rat tail collagen was necessary for the hepatocytes to differentiate and to be maintained on the dishes after 2% DMSO treatment. The cells reached confluence and the surface area of each cell attached to the dish might have decreased. Thereafter, 2% DMSO might help maintain this number of cells and prevent the decrease of cell volume as well as the suppression of cell proliferation. In fact, the height of the cells increased and the area of the lateral membrane attached to the adjacent cells became larger and larger (data not shown). Therefore, the structures of cell-cell adhesions such as desmosomes, gap junctions, and bile canaliculus-like structures developed well after the addition of DMSO (*25-27, 52*). Thus, cell shape may play a crucial role in the maintenance of highly differentiated hepatic functions.

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