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Posttranscriptional Modulation of Gene Expression in Cultured Rat Hepatocytes

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The maintenance of high levels of two liver-specific mRNAs in cultured hepatocytes was achieved in a serum-free hormonally defined cell culture medium. However, this maintenance of liver-specific mRNA levels did not correlate with the level of transcription of the genes but was apparently due to increased stabilization of the tissue-specific mRNAs. The mRNA stabilization did not occur in serum-supplemented medium. In both defined and serum-supplemented medium, actin and tubulin mRNAs were also greatly increased, in both cases predominantly if not entirely due to increased mRNA stability.

One of the crucial problems in cell biology is the analysis of tissue-specific gene regulation in normal differentiated cells. Many studies in which whole tissues and cultured cell lines are used have implicated the rate of transcription of genes as the primary site of regulation of mRNA production for a variety of genes (9, 10). However, factors which may further modulate levels of gene expression are not easily studied in whole tissues. In cultured cells there is usually a decrease or a complete loss of expression of tissue-specific functions (4, 7, 11, 19, 23). Recently, the development of new serum-free hormonally defined media (2, 11, 21) and the use of matrix substrata (25, 28) for culturing differentiated cells have improved the chances of studying tissue-specific gene regulation in primary cultures. In these studies we have compared the transcriptional rates and the cytoplasmic abundance of several mRNA sequences, some encoding common functions and some encoding liver-specific functions in primary cultures of rat hepatocytes plated onto tissue culture plastic and maintained in a serum-supplemented medium (SSM) or a serum-free, hormonally defined medium (HDM).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were purchased from Marland Farms and maintained for 1 week under 12-h light-dark cycles with food and water available ad libitum. Rats weighing between 200 and 275 g were used as the source of hepatocytes.

Preparation of cells. Rat hepatocytes were prepared by the procedure of Berry and Friend (3), using the perfusion mixture of Leffert et al. (19).

Culture conditions. Hepatocytes were cultured in RPMI 1640 medium (GIBCO), supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml (and for day 1 of culture, 250 μg of amphotericin per ml). This medium was supplemented with 10% fetal bovine serum (Sterile System) to produce SSM or with the following mixture of hormones, growth factors, and trace elements to produce HDM: insulin (265 mU/ml), glucagon (10 μg/ml), epidermal growth factor (50 μg/ml), prolactin (2 mU/ml), human growth hormone (10 μU/ml), linoleic acid (5 μg/ml, which was made up in 100× stock in 1 mg of delipidated bovine serum albumin per ml), copper (1×10^{-7} M), selenium (3×10^{-10} M), and zinc (5×10^{-11} M) (11). Isolated hepatocytes were plated and cultured for the first 24 h in HDM plus 10% fetal bovine serum (SSM/HDM). Thereafter cultures were maintained in SSM or HDM. Cultures were fed every 24 h for the duration of the experiment.

Culture protocol. Rat hepatocytes were plated in plastic culture dishes (Falcon Scientific Co.) at 1.5×10^7 cells per 150 cm^2 and cultured for the first 24 h in SSM/HDM and then switched to the test medium (HDM, SSM, or SSM/HDM) as described below.

Nuclear transcription. Cultures (after 2 or 5 days) were washed twice with ca. 50 ml of ice-cold phosphate-buffered saline. Cells were scraped (three to five 150-mm plates per assay) and lysed by Dounce homogenization (ca. 13 strokes with a B pestle) in ice-cold reticulocyte standard buffer containing 0.1% Triton X-100. Whole liver nuclei were prepared from a control rat (to be used as a comparison with the liver cultures) by methods described previously (7). Cell lysis was monitored by phase microscopy. The nuclear RNA labeling, isolation, and hybridization analysis have been previously described (7). DNAs used in hybridization included DNA complementary to mRNA from mouse albumin (17; gift of S. Tilghman, Institute for Cancer Research, Philadelphia), rat albumin (30; from M. Zern, Albert Einstein College of Medicine, New York), α_1-antitrypsin (clone was characterized by K. Krauter, B. Citron, D. Powell, and J. E. Darnell, Jr., manuscript in preparation), and rat β-actin and rat β-tubulin (8; gift of N. Cowan, New York University Medical Center, New York).

mRNA concentrations. Cultures were initiated by seeding 1.5×10^7 cells per tissue culture dish (150 mm; Falcon Scientific Co.) and grown under the culture conditions specified. In each experiment, cells were pooled from two to four dishes per culture condition. Dishes were washed twice with 40 ml of cold phosphate-buffered saline. The cells were removed with a rubber policeman, washed twice in 50 ml of cold phosphate-buffered saline, and pelleted, and total RNA was isolated by the guanidinium-hot phenol method of Feramisco et al. (13). RNA samples (15 μg of total RNA as measured by optical density at 260 nm per lane) were resolved by electrophoresis through 1% agarose submerged-slab denaturing gels. RNA was transferred to GeneScreen (New England Nuclear Corp.) and then prehybridized and hybridized (2×10^6 cpm/ml) by method II in the GeneScreen instruction manual. The cDNA clones complementary to

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specific mRNAs were radioactively labeled by nick translation as described by Rigby et al. (26) and as modified by Rajan et al. (24). [^{32}P]dCTP (specific activity, 3,000 Ci/mmol; New England Nuclear Corp.) was included in the nick-translation reaction to obtain a specific activity of 1.5×10^8 to 4.5×10^8 cpm/μg of DNA.

RESULTS

Cell cultures. The cultures were maintained for up to 5 days, because when plated onto tissue culture plastic and in HDM, the liver cells detach as a viable cell sheet from the culture dish within 6 to 7 days. However, cells cultured in either SSM or SSM/HDM could be maintained in culture on plastic dishes for ca. 2 weeks.

The freshly plated hepatocytes were ca. 90% confluent after 24 h and became completely confluent by 48 h. Therefore, between 24 and 48 h after the initial plating either some cell division occurred or the cells spread to produce the confluent cultures.

The majority of the cells present in all cultures throughout the experimental period were parenchymal-like, and many binucleated cells characteristic of hepatocytes were observed. In HDM, the cultures remained as more than 95% parenchymal cells throughout the lifespan of the cultures. By contrast, cultures maintained in either SSM or SSM/HDM showed a gradual selection for various nonparenchymal cells, including both endothelial cells and fibroblasts. Although selection for nonparenchymal cells could be a serious problem after 8 to 10 days of culture in SSM or SSM/HDM, parenchymal cells still constituted the majority of the total cell population by the end of our experimental period (5 days).

RNA transcription rates. To assay transcription rates of various RNAs, the nuclei of cells were isolated, and nascent RNA was labeled by chain elongation in the presence of [^{32}P]UTP; the labeled RNA was hybridized to DNA dots on filters (7, 16), and RNase-resistant signals were detected by autoradiography. In comparing different cell samples equal amounts of labeled RNA were used for the hybridizations. Hepatocyte nuclei that had been cultured for 24 h in SSM/HDM plus an additional 1 or 4 days in SSM or HDM showed markedly decreased transcription of albumin and α_1-antitrypsin RNAs compared with control nuclei prepared from fresh liver (Fig. 1a and b, lanes 4 to 7). The albumin transcription rate was most dramatically affected by day 5, showing a decrease in comparison with fresh liver cell nuclei of 8-fold in cells cultured in HDM and a decrease of 32-fold for cells kept in SSM (Table 1). Therefore, neither of the culture conditions examined was able to maintain transcription rates of tissue-specific mRNAs at levels seen in fresh liver nuclei.

By contrast to the liver-specific genes, β-actin and β-tubulin genes in cultured liver cells were transcriptionally active at rates approximately equal to those seen in vivo (Fig. 1a and b, lanes 4 to 7; Table 1) after 2 days and after 5 days in culture. Cells cultured in SSM showed two to three times as much transcription for β-actin and β-tubulin as did cells cultured in HDM.

mRNA concentration: stabilizing effect of HDM. To measure the presence and relative abundance of specific mRNAs, Northern blot analysis was used. RNA was isolated, electrophoresed, blotted, and hybridized with nick-translated cloned DNA, and the resulting autoradiographic bands were quantitated by densitometry. Several different times of exposure of the autoradiograms were obtained, and the densitometric scans showed approximately linear development of the signal with time.

Cells were plated in a mixture of HDM and serum and then switched after 24 h to SSM or HDM. After another 24 h, hepatocytes cultured in SSM or HDM contained decreased levels of both albumin and α_1-antitrypsin mRNA relative to levels in intact liver. At this time both β-actin and β-tubulin mRNA levels were dramatically elevated in cells cultured in HDM or SSM. The cells cultured in SSM (2 days in culture) had greatly increased levels of mRNA for β-actin (+1,000-fold) and β-tubulin (+350-fold) compared with the levels in fresh liver samples (Table 1).

By day 5 in culture, cells which had been maintained in HDM for the last 4 days had levels of mRNA for albumin and α_1-antitrypsin that were about threefold greater than those in 2-day cultures (Fig. 1b, lane 3; Table 1). Indeed, albumin mRNA was as abundant in cells after 5 days in culture in HDM as it was in intact liver. However, cells maintained between 2 and 4 days in SSM exhibited no increase in mRNA α_1-antitrypsin and showed a substantial decrease in the level of mRNA for albumin between 2 and 5 days in SSM. The albumin mRNA had declined to a level 250-fold lower than that in liver cells in vivo. β-Actin and β-tubulin mRNA levels were highest in 2-day cultures and decreased by approximately twofold after culture for 5 days.

Time course of changes in mRNA concentration. To determine the time course of events leading to the differences in the concentration of albumin mRNA observed in Fig. 1, hepatocyte cultures were initiated for 24 h in SSM/HDM and then continued in HDM or SSM as previously described. RNA was isolated from rat liver and from cultures at various intervals after plating (24, 72, and 120 h) and analyzed by the Northern blot procedure. Albumin mRNA concentrations decreased during the first 24 h (SSM/HDM) but increased again in HDM at 72 and 120 h, approaching in vivo levels (see Fig. 4). By contrast, cells maintained in SSM after the first 24 h showed a significant decrease in albumin mRNA during the additional 72 h in culture (Fig. 2C, lane 3), and by 120 h (Fig. 2D, lane 3) there was only a barely detectable signal for albumin mRNA. In addition, cells that were maintained in a mixture of HDM plus serum for 120 h (Fig. 2D, lane 2) showed a level of albumin mRNA that was 300-fold less than that in cells held in HDM alone for the same period of time (see Fig. 4). Therefore, the presence of serum in the culture medium seems to block the positive influence of HDM on the maintenance of albumin mRNA levels.

Even after 72 h in one medium, the effects on the mRNA concentration in the liver cells could be changed by switching the type of culture medium used, further indicating that the effects of the medium operate on specific cells and not only through a selection process. When cells were plated, held in SSM for 72 h, and then switched to HDM for another 48 h, the albumin mRNA levels remained similar to those levels found at the time of switching (Fig. 3A). Therefore, the events responsible for the rapid loss of specific mRNA in cultures in SSM can be stopped, and the remaining mRNA can be stabilized. The converse was also true; cells maintained in HDM for 72 h and then switched to SSM for 48 h exhibited an accelerated decrease in albumin mRNA (Fig. 3B and 4).

DISCUSSION

We have described conditions in which changes occur in the concentration of several specific mRNAs that are not correlated with similar changes in transcription rates for those mRNAs. These studies on primary cultures of liver

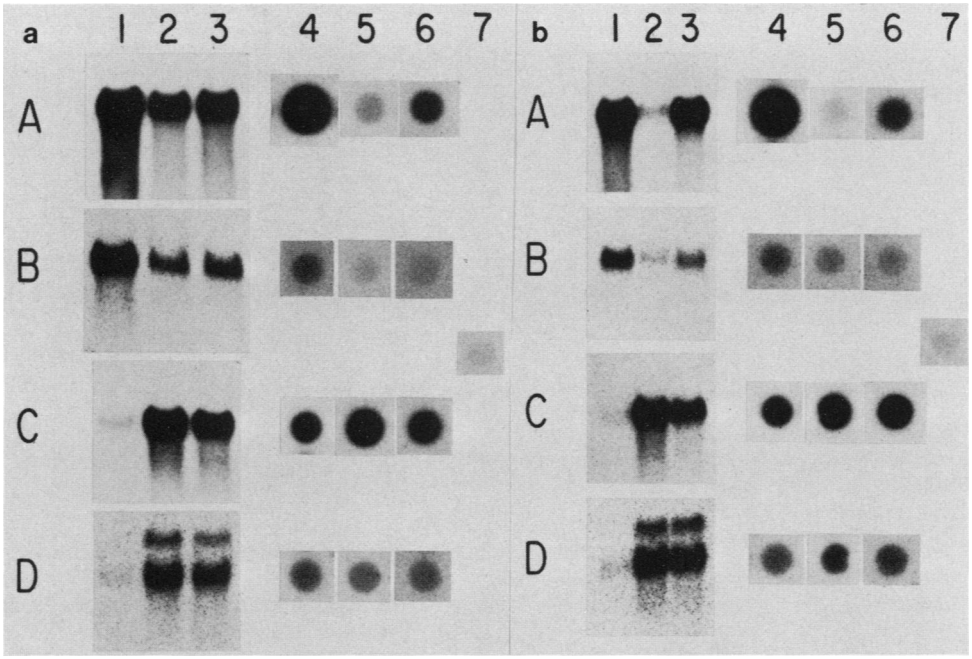

FIG. 1. Comparison of mRNA concentrations and transcriptional rates for albumin (row A), α_1-antitrypsin (row B), β-actin (row C), and β-tubulin (row D) in primary cultures of adult rat hepatocytes grown in culture for 2 days (a) or 5 days (b). The data presented show mRNA concentrations assayed by the Northern blot procedure of mRNA from fresh rat liver (lane 1) and cells maintained in SSM (lane 2) or HDM (lane 3). The transcription assays show dot hybrids (16) of nascent labeled nuclear RNA from rat liver (lane 4), cells in SSM (lane 5), or cells in HDM (lane 6). pBR322 (lane 7) was used as a control for nonspecific hybridization. Densitometric tracings of these data are shown in Table 1.

cells in a serum-free, hormonally defined medium (HDM) provide new evidence that gene expression can be influenced dramatically by posttranscriptional mechanisms which most likely involve changes in mRNA stability. The effects were found to operate on both common and tissue-specific mRNAs. However, the common mRNAs were found to be stabilized by both media tested, whereas the tissue-specific mRNAs were stabilized only in HDM.

First, mRNAs for two common cytoskeletal proteins, β-actin and β-tubulin, seem to be dramatically stabilized in response to culturing, but these changes occurred in both HDM and SSM. The 1,000-fold rise in actin concentration within 2 days is so great that an increase in transcription (soon after cell plating; i.e., before we analyzed the rate) is presumed to have occurred to achieve the observed effect. Indeed, a very strong but brief period of heightened transcriptional activity for actin RNA sequences was noted after disaggregation of mouse liver cells (7). However, in those previous experiments, the rate of actin transcription declined significantly by 7 h and reached a level similar to that

TABLE 1. Effect of different culture conditions on transcription rates and mRNA steady-state levels in adult rat hepatocytes

Functions	Change in cultures relative to rat liver[a]							
	2-day				5-day			
	Relative mRNA steady-state level		Relative transcription rate		Relative mRNA steady-state level		Relative transcription rate	
	SSM	HDM	SSM	HDM	SSM	HDM	SSM	HDM
Liver specific								
Albumin	−3.9	−3.1	−19.1	−9	−250	1	−32	−8
α_1-Antitrypsin	−37	−17	−3.3	−4.3	−30	−6	−2.6	−3.3
Common								
β-Actin	+1,000	+450	+3	+1.4	+690	+220	1	1
β-Tubulin	+350	+220	1	1	+120	+78	−2	−4

[a] Data presented as change relative to rat liver, e.g., −3.9 is 3.9 times less than rat liver. Transcription data were from experiments in which equal amounts of RNA (±20%) from different samples were used. From densitometric analysis of autoradiographs, a small correction based on the 28S rRNA density was made after nonspecific hybridization assessed with pBR322 DNA was substracted. An average of two different control rat liver transcriptions was used to calculate the 2- and 5-day data. mRNA concentrations were determined by densitometric scans of Northern blots exposed for different lengths of time to compare very abundant with less abundant mRNA signals.

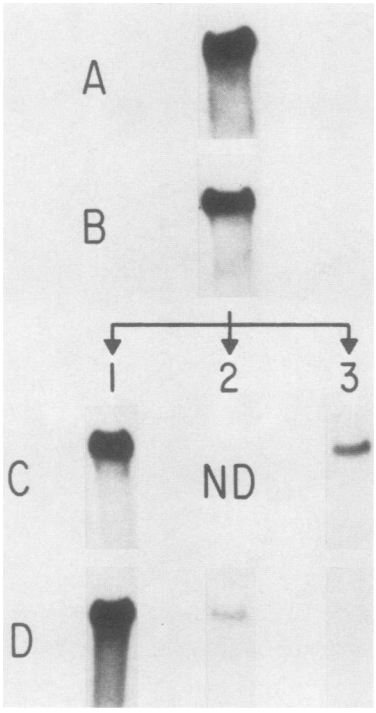

FIG. 2. Demonstration of the effect of different culture conditions on the steady-state albumin mRNA levels as measured by Northern blot analysis (12). Cells were isolated from adult rat liver, and RNA was isolated after 0 (A), 24 (B), 72 (C), and 120 (D) h in culture. For the first 24 h of culture, cells were maintained in HDM plus serum after which they were switched to HDM (lane 1), HDM plus serum (lane 2), or SSM (lane 3). ND, Not determined.

for liver in vivo within 24 h of cell plating. In those experiments, no substantial increase in transcription of actin mRNA or in abundance of actin mRNA was noted for 24 h. In the present experiments, we did not analyze transcription before 24 h of culture. At 48 h, we also found that transcription in cultures was equal to that in cells in vivo. Therefore, the increased levels of actin and tubulin mRNAs observed in the cultures after 24 h must also be due to a posttranscriptional mechanism(s) influencing mRNA abundance. This posttranscriptional effect may be related to changes in cell shape and cell anchorage, as has been earlier suggested for tubulin mRNA in fibroblasts (12).

Changes in the cytoplasmic concentration of two tissue-specific mRNAs (albumin and α_1-antitrypsin) seem to be dependent on components in the culture medium. The presence of serum is accompanied by an apparent decreased stability, whereas a hormonally defined medium supports an mRNA stability that could be greater than that in normal intact liver. For example, the albumin mRNA concentration increased between 24 and 120 h in culture in HDM to in vivo levels despite a consistently low level of transcription by cells in this medium.

The most probable posttranscriptional mechanism responsible for regulating the abundance of the mRNAs assayed in the liver cultures is mRNA stabilization. Hormonal regulation of mRNA stability has been documented in several systems (D. J. Shapiro and M. L. Brock, *in* G. Litwack, ed., *Biochemical Actions of Hormones*, vol. 14, in press). Guyette et al. (15) were first to show that in mammary cultures a protein hormone, prolactin, could alter a tissue-specific function, casein secretion, in mammary cultures by affecting mRNA stability. Brock and Shapiro (5) showed that estrogen can stabilize vitellogenin mRNA in cultured *Xenopus* liver. Similarly, Laverriere et al. (18) have shown that thyroliberin increases the half-life of prolactin while reducing the stability of growth hormone mRNA. Cyclic AMP has been shown to specifically stabilize *Dictyostelium* mRNAs

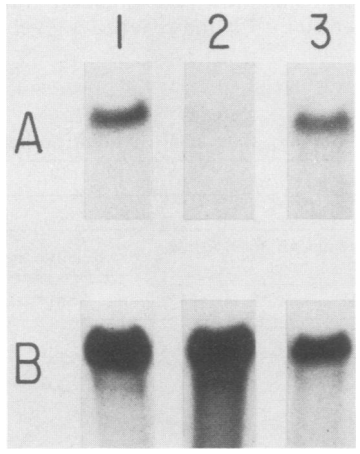

FIG. 3. Northern blot analysis, showing reversibility of albumin mRNA loss. After the initial 24-h plating period in HDM plus serum, cells were cultured in SSM (row A) or HDM (row B) for a total culture period of 72 h (lane 1) and 120 h (lane 2). Some of the cells were switched from SSM at 72 h to HDM for another 48 h (row A, lane 3). Conversely, a portion of cells grown in HDM for 72 h were switched to SSM for an additional 48 h (row B, lane 3).

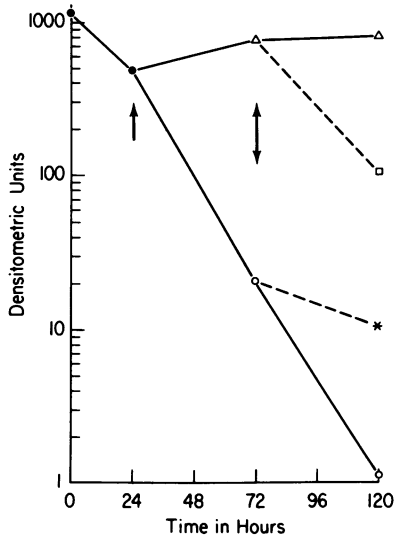

FIG. 4. Graphic representation of densitometric values for Northern blot data for albumin mRNA presented in Fig. 2 and 3. The value for fresh rat liver is at 0 h, and the arrow at 24 h shows the time point at which the cell medium was changed from the original conditions of HDM plus serum to HDM (△) or SSM (○); the resulting levels of albumin mRNA are represented by the solid lines (data from Fig. 2). The arrow at 72 h indicates the time point at which the medium in some of the dishes was switched to the opposite condition (SSM to HDM [*] or HDM to SSM [□]), resulting in the changes shown for albumin mRNA levels as indicated by the dashed lines.

which are developmentally regulated (6, 20). Adenovirus mRNAs (transcriptional units 1A and 1B) are differentially stabilized during the virus growth cycle (29). Histone mRNA stability varies in *Drosophila* embryos during embryogenesis (1) and in sea urchin embryos during cleavage (22, 27). Also, there is a substantial increase in RNA stability starting at the time of germinal vesicle breakdown in the *Xenopus* oocyte (14). The mechanism(s) by which the stability of mRNA is regulated by a specific hormonal milieu or by other conditions is unknown in any of these systems.

In the rat liver cells used in these experiments and cultured on tissue culture plastic and in any of the media tested, transcription rates for liver-specific mRNAs were significantly decreased from in vivo rates, as was earlier described for mouse liver (7). Eight liver-specific genes in addition to the two shown in Fig. 1 were assayed with the available mouse liver cDNAs (7), and all gave reduced signals in cultured rat cells (data not shown). These results agree with and extend the previous report of Clayton and Darnell (7), showing that transcription of liver-specific functions decreased dramatically within hours when the liver cells were cultured in SSM. Clearly, some factor(s) required for continued transcription at levels approximating those in vivo has been destroyed or altered when cells are cultured in SSM.

These studies on rat liver cultures add to and strengthen the growing realization that differentiation of cells is also regulated by a posttranscriptional mechanism(s) such as regulation of mRNA stability and indicate that such a mechanism(s) is likely to be far more common and more significant than heretofore realized. A new discovery from these studies is that primary cultures of rat liver cells can be used to analyze the posttranscriptional regulatory mechanism(s) under completely defined conditions. The use of totally defined culture conditions should aid in defining factors (hormones, growth factors, and trace elements) that play a role in the regulation of mRNA stability and other posttranscriptional mechanisms affecting mRNA abundance. These include both the mechanisms that result in decreased or increased abundance of specific mRNAs.

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