

## Dependence of Liver-Specific Transcription on Tissue Organization

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**When the liver is disaggregated and hepatocytes are cultured as a cellular monolayer for 24 h, a sharp decline (80 to 99% decrease) in the transcription of most liver-specific mRNAs, but not common mRNAs, occurs (Clayton and Darnell, *Mol. Cell. Biol.* 2:1552-1561, 1983). A wide variety of culture conditions involving various hormones and substrates and cocultivation with other cells failed to sustain high rates of liver-specific mRNA synthesis in cultured hepatocytes, although they continued to synthesize common mRNAs at normal or elevated rates. In contrast, when slices of intact mouse liver tissue were placed in culture, the transcription of liver-specific genes was maintained at high levels (20 to 100% of normal liver). Furthermore, we found that cells in the liver could be disengaged and immediately reengaged in a tissue-like structure by perfusing the liver with EDTA followed by serum-containing culture medium. Slices of reengaged liver continued to transcribe tissue-specific mRNA sequences at significantly higher rates after 24 h in culture than did individual cells isolated by EDTA perfusion followed by culturing as a monolayer. Therefore we conclude that a mature tissue structure plays an important role in the maintenance of maximum tissue-specific transcription in liver cells.**

The liver offers many advantages in the study of cell differentiation. A single cell type, the hepatocyte, constitutes over 90% of the mass of the organ (13), which in adult mammals accounts for 1% or more of total body weight (9). The liver from a single 30-g mouse, for example, contains more than  $10^8$  hepatocytes (4). Furthermore, unlike many other differentiated tissues, the architecture of the liver is uniform throughout the organ: hepatocytes, linked by tight junctions and contacting an extracellular matrix, are arranged in plates separated by sinusoids (frequently fenestrated capillaries) that are lined by rather scarce endothelial cells (19). This architecture in the adult reflects a continuation of the interaction of endodermal and mesodermal components that begins with tissue induction (16, 26). Finally, more cell-specific protein products (intracellular enzymes and secreted proteins) are known for hepatocytes than perhaps any other cell type (20), and the molecular basis for cell-specific gene control is now a central issue in developmental biology.

To study liver-specific gene control we have selected cDNAs that are complementary to a series of mRNAs present in liver, but not in brain and cultured cells (8). With this material it was shown that transcriptional activation underlies the production of most liver-specific mRNAs. This conclusion was reached by comparing liver with other tissues (8, 36) and by examining the liver from about 14 days of gestation to adulthood (36). Also, cells regenerating liver were found to continue high rates of liver-specific mRNA synthesis (11), implying that liver-specific gene control is not tightly linked to control of the cell cycle and replication.

Evidence of specific signals or mechanisms which coordinate the transcription of liver-specific genes in the adult organ has come from experiments with primary cultures of hepatocytes (4). When the liver was disaggregated and the hepatocytes were cultured as a cellular monolayer for 24 h, a sharp decrease was observed in the transcription of most liver-specific mRNA sequences, but not of mRNAs common to all cells. The cells do continue to make liver-specific RNAs, but at a greatly reduced rate. Thus something critical for the maximum rate of transcription for most liver-specific

genes must be lost when hepatocytes are dispersed and cultured. This critical element was presumed to involve either tissue organization (cell contacts, shape, or structure) or soluble components of the humoral environment (nutrients or hormones and other factors).

In this paper, we examine further the decrease of tissue-specific transcription that occurs in hepatocytes when they are cultured as cell monolayers. Various alterations in the culture medium do not sustain specific transcription at high rates. However, when hepatocytes are cultured as slices of tissue where cell contact and cell architecture are maintained, tissue-specific transcription is maintained at a high rate (20 to 100% of the maximal rate). The cells in slices continue the high rate of liver-specific RNA synthesis in culture medium lacking added hormones or serum. Thus tissue organization, and not nutritional conditions, appears to be most important in maintaining a high rate of liver-specific transcription for the 24-h period examined.

Another separate line of research, examination of liver-specific products in hepatoma cell lines (reviewed in reference 45), has also provided evidence for mechanisms of coordinate control of liver-specific genes. In the accompanying paper (5) we compare liver-specific mRNA production in various hepatoma cell lines, and from the results of these two sets of experiments we conclude that liver-specific transcription can be activated to a low level of expression that continues in isolated cells, but a high level of transcription requires that hepatocytes be organized in mature, intact tissue.

### MATERIALS AND METHODS

**Preparation of primary hepatocyte cultures.** Hepatocytes were isolated from C57/B6 mouse livers by perfusion with EDTA followed by collagenase (Worthington Diagnostics; class II) at 100 U/ml (4). Hepatocytes were released when EDTA was omitted from the first perfusion step, but with lower viability and yield (about 40% viability of  $7 \times 10^7$  cells). Where appropriate (see below), other constituents (insulin, hydrocortisone, testosterone, cycloheximide, concanavalin A, demecolcine, cytochalasin B) were added at the concentrations given below to the perfusate or to the initial cell suspension. Cells were plated in the appropriate culture

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TABLE 1. Recombinant cDNA-containing plasmids in these experiments

Function	Abbreviation <sup>a</sup>	Encoded product	Source	Plasmid designation	Donor or reference
Liver Functions	1	Major urinary protein	Mouse	plivS-1	Derman et al. (8)
	2	Unknown	Mouse	plivS-2	Derman et al. (8)
	3	$\alpha$ -1-Antitrypsin	Mouse	plivS-3	Derman et al. (8)
	4	Unknown	Mouse	plivS-4	Derman et al. (8)
	5	Unknown	Mouse	plivS-5	Derman et al. (8)
	6	Transferrin or related	Mouse	plivS-6	Derman et al. (8)
	9	Unknown	Mouse	plivS-9	Derman et al. (8)
	10	Unknown	Mouse	plivS-10	Derman et al. (8)
	Al	Serum albumin	Mouse	pmalb2	Kioussis et al. (22)
	Common Functions and Controls	p	(Plasmid vector)		pBR322
Ac		$\beta$ -Actin	Chicken	pA1	Cleveland et al. (6)
aT		$\alpha$ -Tubulin	Human		P. A. Sharp
bT		$\beta$ -Tubulin	Rat		N. Cowan
B		Unknown	Chinese hamster	pCHO-B	Harpold et al. (15)
tR		tRNA-arginine	<i>Xenopus laevis</i>	pyH48	D. Brown
28S		28S rRNA	Mouse		N. Arnheim

<sup>a</sup> These abbreviations are used in the keys to the figures and tables. The encoded products of plivS-3 ( $\alpha$ -1-antitrypsin) and plivS-6 (transferrin) have recently been identified (manuscripts in preparation).

medium on 150-mm (Falcon) or 100-mm (Nunc) plastic tissue culture dishes or (as indicated below) on bacterial petri dishes covered with biomatrix substrate (10, 38) that were prepared and provided by L. Reid and D. Jefferson. Cells were routinely plated at a density of  $6 \times 10^4/\text{cm}^2$ , although density could be varied between  $2 \times 10^4$  and  $16 \times 10^4$  cells per  $\text{cm}^2$  (33) with a minimal effect on the transcriptional profile. Cells were maintained in humidified incubators (5%  $\text{CO}_2$  in air) or under a humidified atmosphere of 5%  $\text{CO}_2$  in  $\text{O}_2$  at 37°C. Attached cells were rinsed and given fresh medium after 2 h in culture. For suspension cultures, hepatocytes were placed in Erlenmeyer flasks on a rotary shaker (50 to 100 rpm) at 37°C under an atmosphere of 5%  $\text{CO}_2$ -95%  $\text{O}_2$ .

Hepatocytes were released from the liver without using collagenase by retrograde perfusion (4), typically for 1 h with HEH, which contained the following (per liter): 400 mg of KCl, 60 mg of  $\text{KH}_2\text{PO}_4$ , 8,000 mg of NaCl, 90 mg of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 1,000 mg of dextrose, 17 mg of phenol red, 10 mM EDTA, and 5 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) (pH 7.3). The flow rate was maintained at 7 ml/min with a peristaltic pump, the temperature was maintained at 37°C, and a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  was bubbled slowly through the perfusate reservoir. After dispersion and centrifugation, the cells from one liver were suspended in the perfusing solution (12 ml) followed by the addition of 13.7 ml of isoosmotic Percoll (Pharmacia Fine Chemicals; made by adding 1 part of 1.5 M NaCl to 9 parts of Percoll), to result in a solution density of 1.07 g/ml. The suspension was centrifuged at room temperature for 30 min ( $1,500 \times g$ ) to yield a pellet of approximately  $3 \times 10^7$  cells (>95% viable by trypan blue exclusion).

Fetal liver cells were liberated by incubating minced fetal livers in the collagenase solution used to isolate hepatocytes (above) at 37°C for about 1 h with several changes.

**Cell culture media and additives.** For basal culture conditions, cells were cultured in Dulbecco minimal essential medium with insulin (Iletin I; Eli Lilly & Co.) and hydrocortisone sodium succinate (Solu-Cortef; The Upjohn Co.) added at 10 mg/liter each, heat-inactivated fetal bovine serum (Sterile Systems) added to 10%, and gentamicin (Schering Corp.) added to 50 mg/liter. Non-heat-inactivated serum had no different effect on the visible appearance of cultures or on transcription. Other alterations of the basal

medium included the addition of the following: horse serum (Sterile Systems) at 10%, testosterone (Sigma Chemical Co.) at  $10^{-6}$  to  $10^{-7}$  M, epidermal growth factor (Sigma) at 2 mg/liter, glucagon (Sigma) at 10 mg/liter, all-*trans* retinoic acid (Eastman Chemical Products, Inc.) at  $10^{-7}$  M, dimethyl sulfoxide (Sigma) at 1.5%, dibutyryl cyclic AMP (Sigma) at  $10^{-4}$  to  $10^{-3}$  M, sodium butyrate at 5 mM, cycloheximide (Sigma) at 22 mg/liter, concanavalin A (Calbiochem) at 25 mg/liter,  $\text{CaCl}_2$  at 6 mM, A23187 (Calbiochem) at 1 mg/liter with dimethyl sulfoxide at 1.5%, sodium orthovanadate at 10  $\mu\text{M}$ , cytochalasin B (Sigma) at  $2 \times 10^{-5}$  M plus 0.5% dimethyl sulfoxide, and demecolcine (Sigma) at 1  $\mu\text{M}$ . The hormonally defined medium of Enat et al. (10) was provided by L. Reid and D. Jefferson and contains the following: RPMI medium with penicillin (100 U/ml), 100 streptomycin ( $\mu\text{g}/\text{ml}$ ), insulin (265 mU/ml), glucagon (10  $\mu\text{g}/\text{ml}$ ), epidermal growth factor (50  $\mu\text{g}/\text{ml}$ ), prolactin (2 mU/ml), human growth hormone (10  $\mu\text{U}/\text{ml}$ ), linoleic acid (5  $\mu\text{g}/\text{ml}$  with delipidated bovine serum albumin [BSA], 1  $\mu\text{g}/\text{ml}$ ), copper ( $10^{-7}$  M), selenium ( $3 \times 10^{-10}$  M), and zinc ( $5 \times 10^{-11}$  M). Arginine-free ornithine-supplemented basal medium of Leffert et al. (27) and Williams D medium were obtained from GIBCO Laboratories and were supplemented with insulin, hydrocortisone, and serum as described above for basal medium. Hepatocytes were infected with adenovirus 5 by addition to the medium of 5,000 particles per cell after 36 h in culture (this resulted in cell death about 2 days later, and transcription of the E1A gene was robust by 6 h postinfection). Liver extract was prepared by polytron homogenization in the cold of adult mouse livers in Dulbecco minimal essential medium and clarification by centrifugation (5 min at  $1,000 \times g$ ); supernatants were prepared from a total of six livers in three installments over 24 h and added immediately to the culture medium (36 ml) of  $3 \times 10^7$  hepatocytes (about one-third of one liver).

For most long-term cultures the medium was changed every 1 or 2 days, except in experiments with arginine-free medium when the medium was not changed after initial cell attachment and rinsing (27).

**Coculture of hepatocytes with other cell types.** Rat liver epithelial cells were provided by C. Guguen-Guillouzo and cocultured with mouse hepatocytes as described previously (14). Bovine aortic endothelial cells were provided by E.

Jaffe and seeded at a 1:1 ratio onto attached hepatocytes after 2 h in culture. Mouse liver nonparenchymal cells were centrifuged at a high speed ( $500 \times g$  for 10 min) from the supernatant after freshly dissociated hepatocytes had been pelleted at low speed, yielding about  $3 \times 10^7$  nonparenchymal cells from one mouse liver; 1 to 10 times as many nonparenchymal cells were plated along with fresh hepatocytes.

**Liver slice preparation and culture.** Livers to be sliced were first perfused with about 20 ml of Dulbecco modified essential medium or basal culture medium (see above). Liver lobes were arranged flat side down on the chopping platform of the McIlwain Tissue Chopper (Brinkmann Instruments, Inc.) and were cut twice at right angles. Although a slicing thickness of 0.325 mm was usually used, thickness could be varied between 0.150 and 0.450 mm with negligible effect on specific gene transcription in incubated slices. Reengaged livers (described below) were cut at a thickness of 0.450 mm, although the resulting fragments were approximately 0.200 mm in diameter. Newly prepared slices were separated from one another by pipetting in basal medium and were washed in several changes of basal medium before culturing began. Slices from one liver or the equivalent were placed in two 500-ml Erlenmeyer flasks with 70 to 100 ml each. A constant stream of the humidified gas mixture (5%  $\text{CO}_2$  in  $\text{O}_2$ ) was introduced into the flasks throughout the culture period. The rotary shaking rate was adjusted (usually 70 to 80 rpm) so the slices spread evenly or formed a slowly revolving ring at the bottom of the flask (clumping in the middle and washing up on the side were avoided). The medium was changed first at 2 h and typically every 4 to 8 h thereafter by gravity sedimentation in the culture flasks and aspiration of the supernatant.

**Recombinant plasmids.** The plasmids used in these experiments are described in Table 1. Plasmids were grown in *Escherichia coli* strains, harvested, and purified as described previously (28).

**Isolation of nuclei.** Nuclei were isolated for transcription rate analysis by Dounce homogenization or pelleting through a sucrose cushion (4). Nuclei were typically isolated from washed slices by Dounce homogenization in reticulocyte standard buffer with 0.2% Triton X-100 (4).

**Preparation of nascent-labeled nuclear RNA.** Isolated nuclei were washed in an ice-cold buffer of 20 mM Tris (pH 8.1 at room temperature), 20% glycerol, 140 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , and 14 mM 2-mercaptoethanol. The nuclei were suspended and incubated for 12 min at 30°C in 1 to 3 volumes of the same buffer (pH 7.9), but with the addition of [ $\alpha$ - $^{32}\text{P}$ ]UTP (New England Nuclear Corp.; 007H) to 1 mCi/ml, phosphocreatine to 10 mM, and creatine kinase to 100  $\mu\text{g}/\text{ml}$ . Nuclei were lysed by diluting the reaction (typically 0.3 to 1.0 ml) with 1.5 ml of 0.5 M NaCl–0.01 M Tris [pH 7.4]–0.05 M  $\text{MgCl}_2$ –0.002 M  $\text{CaCl}_2$  and pipetting. RNase-free DNase (200 to 400 U; Worthington Diagnostics) was added, and the reaction was held at room temperature with pipetting for about 1 min. Sodium dodecyl sulfate (to 1.5%) and EDTA (to 30 mM) were added, and the reaction was diluted by the addition of 4.5 ml of 10 mM EDTA–10 mM Tris (pH 8.4)–0.5% sodium dodecyl sulfate. After extraction with phenol-chloroform-isoamyl alcohol (24:24:1) and chloroform, RNA was precipitated twice from a large volume (6 to 8 ml) at  $-20^\circ\text{C}$  with yeast tRNA carrier (10  $\mu\text{g}/\text{ml}$ ), 0.2 M NaCl, and 2.5 volumes of ethanol.

Incorporated radioactivity was measured by scintillation counting after spotting samples on DE81 paper and washing several times (5 min each), first in a solution of dibasic

TABLE 2. Densitometric analysis of autoradiographs from transcription experiments

Probe	Absolute density <sup>a</sup>			Avg	Maximum error (+%)
	High input, low exposure	Low input, high exposure	Low input, low exposure $\times 4.73$		
1	21.3	21.5	24.0	22.3	4.5
2	18.8	19.8	21.2	19.9	6.5
3	13.2	13.5	14.2	13.6	4.4
4	2.0	1.8	1.9	1.9	5.0
5	1.7	1.8			
6	1.1	1.1			
9	2.6	2.4	2.4	2.5	4.0
A1	11.7	10.4	9.5	10.5	11
Ac	5.4	5.3	3.8	4.8	21
aT	1.6	1.5	0.9	1.3	30
tR	23.6	23.6	27.4	24.9	10
p	0	0	0		

<sup>a</sup> The densities of dots on autoradiographs were determined by optical scanning and integration; units are in a linear scale, defined by the densitometer. The numerical values for the low-input, low-exposure combination (Fig. 1A) were multiplied by 4.73 to give numbers comparable to the high-input, low-exposure or low-input, high-exposure combinations. Signals for plivS-5 and -6 were not detectable with this combination. Many of the signals from the high-input, high-exposure combination (Fig. 1D) were too dark for densitometric measurement. Maximum error is the largest deviation from the mean of the three measurements, expressed as a percentage of the mean.

sodium phosphate (50 g/liter) and sodium pyrophosphate (8.9 g/liter), then in dibasic sodium phosphate (50 g/liter) alone, and then briefly once each in distilled water and 95% ethanol. Incorporation of approximately  $1.0 (\pm 0.5)$  cpm per nucleus was usually achieved with nuclei from mouse liver and slice cultures, and two to three times as much incorporation was achieved with nuclei from monolayers after 24 h of culture.

**Hybridization of nuclear RNA to plasmid DNA.** Recombinant plasmid DNAs (Table 2) were applied to nitrocellulose filters in 5- $\mu\text{g}$  dots and baked (22, 43). A dot of plasmid vector DNA (pBR322) was always included as a measure of nonspecific hybridization. Replicate filters were prehybridized for 2 h at 65°C in a solution containing  $2 \times$  TESS [50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 300 mM NaCl, 10 mM EDTA, and 0.2% sodium dodecyl sulfate (pH 7.4)] plus 1 mg of yeast tRNA per ml, 0.2% Ficoll, 0.2% polyvinyl-pyrrolidone, and 1.0% sodium pyrophosphate. Labeled nuclear RNA was suspended in a buffer containing  $2 \times$  TESS plus 0.1 mg of tRNA per ml, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.1% sodium pyrophosphate. Samples to be compared were brought to the same concentration of radioactivity by adjusting solution volumes, and 1 to 2 ml of each solution was applied to a prehybridized replicate filter. A 1,000- or 2,000-fold dilution was used for measuring 28S rRNA in a separate hybridization. Filters were hybridized under Paraffin oil at 65°C with rotary shaking for 35 to 40 h. Filters were washed in  $2 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and treated for 1 h at 37°C with pancreatic RNase (0.4  $\mu\text{g}/\text{ml}$ ) and T1 RNase (10 U/ml) followed by proteinase K (50  $\mu\text{g}/\text{ml}$  in 150 mM NaCl–1 mM EDTA–1 mM Tris [pH 7.4]–1% sodium dodecyl sulfate) for 1 h at 37°C, with rinses (in  $2 \times$  SSC) after each treatment.

**autoradiography and densitometry.** Washed hybridization filters were exposed to preflashed (25) Kodak XAR-5 film at  $-70^\circ\text{C}$  with intensifying screens. After development, signal intensity was measured with a Helena Quick-Scan Jr. densitometer. In the course of numerous experiments, the

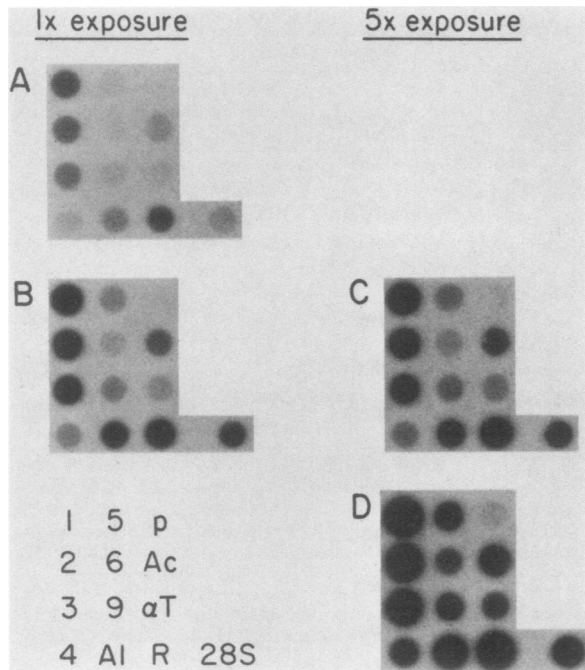


FIG. 1. Linearity of transcription assay for variations in RNA input and autoradiographic exposure time. Plasmid DNAs were dotted on nitrocellulose filters and hybridized to nascent-labeled RNA from fresh mouse liver nuclei prepared by tissue slicing and Dounce homogenization (Materials and Methods). Autoradiographs of the filters after hybridization and washing are shown. Two hybridizations were performed with different inputs of the same labeled RNA sample, and two exposures of the filters to preflashed X-ray film were taken to result in the four images shown. Panels: (A and C)  $2.5 \times 10^6$  cpm of labeled nuclear RNA was hybridized to the filter; (B and D)  $11.8 \times 10^6$  cpm of labeled nuclear RNA (equivalent to that isolated from two-thirds of one liver) was hybridized to the filter. The 1 $\times$  exposure (A and B) was for 3.8 h, and the 5 $\times$  (4.73) exposure (C and D) was for 18 h. The key shows the arrangement of plasmid DNA dots on the filters. (Table 2 gives quantitation of these autoradiographic signals.)

relative transcription rate of RNA complementary to CHO-B was consistently the same in different preparations of hepatocyte nuclei from tissues or cultured cells (unpublished observations), and we have found the signal for CHO-B to be useful as an internal standard of hybridization. To compare quantitations of hybridizations using different inputs of radioactivity, and to correct for any small variations in hybridization conditions or specific activity of labeled RNAs, quantitative data from densitometric analysis (Tables 4 and 7) were normalized to set the CHO-B signal equivalent in all samples.

## RESULTS

**Quantitative assay of specific mRNA transcription rates.** To measure the rate of transcription of specific genes in different populations of cells, we isolated nuclei from the cells and allowed already initiated RNA chains to be elongated in the presence of [ $^{32}$ P]UTP. Under the conditions used, the amount of labeled UTP incorporated into RNA is proportional to the number of polymerases active on each primary transcription unit at the time of nuclear isolation (7). Equal amounts of radiolabeled RNA from the different samples of nuclei were hybridized to cloned DNA samples (see Table 1) deposited as dots of DNA on nitrocellulose (21). The DNA

samples included some that are complementary to liver-specific mRNAs and others that are complementary to mRNAs that are common to many cell types. The amount of hybridization was assayed by autoradiography of RNase-resistant hybrids followed by densitometric scanning. The relative transcriptional activity of each liver-specific gene could thus be compared, both between different samples and to the relatively constant transcription of the common genes.

To determine the quantitative accuracy of this dot-hybrid autoradiographic technique, the experiment shown in Fig. 1 and Table 2 was conducted. The quantitative accuracy of this assay is dependent upon two factors. First, plasmid DNA probes should be in sufficient excess to their complementary RNAs so that the amount of each RNA hybridized will be directly proportional to its concentration in the hybridization solution. Second, the density of the autoradiographic image on film should be in direct proportion to the product of exposure time and radioactive signal intensity. The experiment shown in Fig. 1 and Table 2 shows that both of these conditions are met for the procedures we have used. Two amounts of nascent-labeled mouse nuclear RNA were hybridized to filters containing plasmids representing the full range of signal intensities encountered in these experiments. Decreasing the amount of RNA normally used in the hybridization reaction by a factor of about 5 (4.73) resulted in an equivalent decrease in signal intensity for all of the plasmids

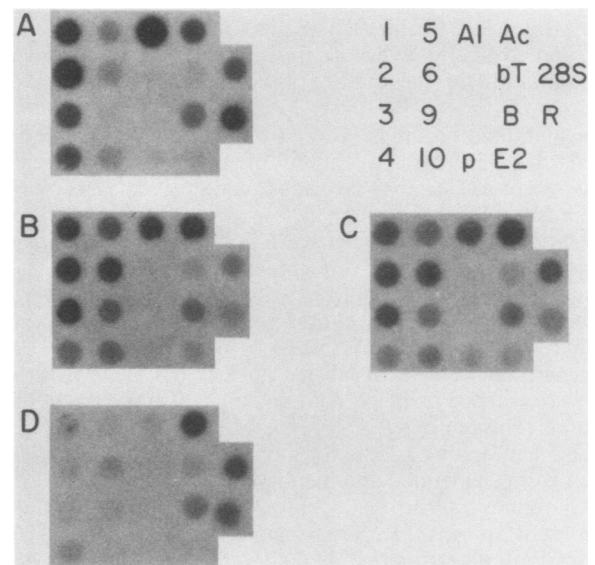


FIG. 2. Transcription rate analysis of liver slice cultures compared with normal liver and monolayer cultures. Autoradiographs of plasmid DNA-containing filters prepared and hybridized as in Fig. 1. Equal counts of nascent-labeled nuclear RNA from mouse liver (A), mouse liver slices cultured in basal medium (B), slices cultured in basal medium with no added hydrocortisone or insulin (C), and isolated hepatocytes cultured in basal medium (D) were hybridized to replicate filters. Liver slices and isolated hepatocytes were prepared as described in the text and cultured for 24 h under an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> with a total of five changes of culture medium each. The combined slices from three livers were split into three equal samples for B, C, and a third culture (not shown; described in footnote *a* of Table 5). The plasmid DNA probes used (Table 1) were arranged on the filters as indicated in the key at the upper right-hand corner. The probe for the E2 gene of adenovirus 5 was included for other reasons, but indicates the presence of a cross-reacting RNA in mouse liver cells that has been observed in other experiments (unpublished observations).

(Table 2). Thus, the hybridization signal varied linearly with the input of a single preparation of RNA.

When the exposure time of the filter from the hybridization reaction with lower input (Fig. 1C) was increased by about fivefold, the autoradiographic image was indistinguishable from that obtained with the approximately fivefold-higher input of RNA and short exposure (Fig. 1B). The weakest signal that could be read with confidence at the lowest exposure (Fig. 1A) was about 1/20 of the strongest; since the strongest signal increased proportionately with a 5-fold increase in exposure time (Fig. 1C), we were able to obtain a linear measure of signal intensities over a range of at least 100-fold. The only significant departures from linearity were observed with very weak hybridization signals in very short exposures (for example, probes 5 and 6 in the low input-short exposure combination gave no detectable autoradiographic signal; Table 2). For densitometric analysis of very low signals, therefore, we always used exposures in which the signal for pBR322, representing the background of nonspecific hybridization, was detectable. As shown in Table 2, errors of  $\pm 4$  to 11% were estimated in measurements of moderately to intensely transcribed genes (plivS-1 through -4 and -9, albumin) and errors of  $\pm 30\%$  for genes giving weak transcriptional signals (actin,  $\alpha$ -tubulin).

**Loss of liver-specific transcription in isolated hepatocytes.** Starting from the previous observation that liver-specific gene transcription in hepatocytes declines during the first 24 h of primary culture (4), we first sought culture conditions which would halt or reverse this decline. Freshly isolated cells were cultured under many different conditions, including the routine culture conditions (10% fetal bovine serum in

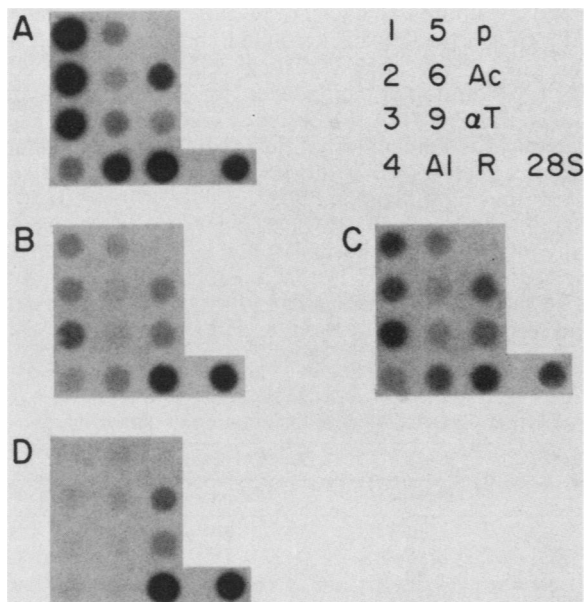


FIG. 3. Transcription in stationary versus shaking slice cultures. Autoradiographs of filters hybridized with nascent-labeled nuclear RNA from mouse liver (A), shaking cultures of slices (B), slices cultured on a stationary platform (C), and isolated cultured hepatocytes (D) are shown. (B) One-half of the slice preparation from two livers was cultured in basal medium as described in the legend to Fig. 2. (C) The other half of the slice preparation was placed on stacks of sterilized Whatmann 3mm filter papers in four 150-mm tissue culture dishes, with basal culture medium added until it just reached the top piece of paper; dishes were cultured in incubators under 5%  $CO_2$ -95% air.

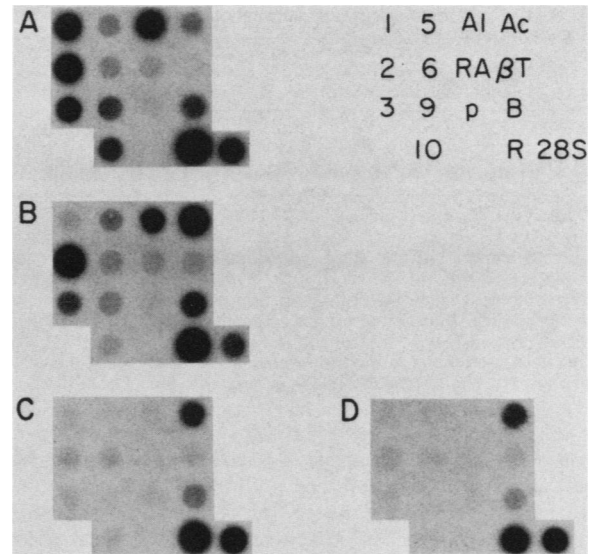


FIG. 4. Transcription in hepatocytes dispersed with EDTA versus collagenase. Autoradiographs are shown of filters hybridized with equal amounts of radiolabeled nuclear RNA isolated from mouse liver (A), from hepatocytes dispersed with EDTA alone followed by 2 h of culture (B) and 24 h of culture (C), and from hepatocytes dispersed by the conventional collagenase perfusion technique followed by 24 h of culture (D).

Dulbecco minimal essential medium plus insulin and hydrocortisone, with the cells plated on tissue culture plastic). Typically after 24 h of culture, specific gene transcription rates were compared in nuclei from the various cultures and in normal livers.

Changes in culture conditions (see Materials and Methods) included alterations in the basal medium, the amount and type of serum and hormones added, cell density, exposure to oxygen, the addition of physiological and nonphysiological inducers and drugs, the inclusion of other cell types in the cultures, and the plating of cells on protein-coated dishes (biomatrix) (10). Culture times were varied from 1 to 10 days, and the response of fetal liver cells was measured. Culture conditions with different reported effects on cell replication were also examined (10). The experimental result was inevitably the same: dispersed cells cultured in monolayers or in suspension showed greatly decreased rates of liver-specific transcription (Fig. 2D, 3D, and 4D). Indeed, isolated cells exposed to any of the culture conditions described in Materials and Methods had similar transcription profiles for the panel of tissue-specific mRNAs.

**Preparation and culture of liver slices.** If tissue-specific transcription in the liver depends somehow on normal tissue organization, then transcription might be maintained in vitro if tissue organization could be maintained in vitro. To that end, we explored the use of conventional tissue-slicing techniques (1, 30) as a way to prepare tissue for culture. Livers to be sliced were first perfused with tissue culture medium to wash out blood and to insure quick exposure of all of the cells in the tissue to the culture medium. With a McIlwain tissue chopper, livers were then cut into columns 0.325 by 0.325 by 2 to 3 mm. The columns were therefore approximately 10 to 15 cells in diameter, or about half the size of the classic liver lobule (19). In most experiments the slices were placed in Erlenmeyer flasks that were flushed

TABLE 3. Macromolecular synthesis in liver slice cultures<sup>a</sup>

Time (h)	RNA				Protein			
	cpm	mg	cpm/mg	%	cpm	mg	cpm/mg	%
2	1,452	1.8	807	100	113,300	2.0	56,650	100
26	1,713	1.6	1,070	133	63,800	1.4	45,570	80
50	1,030	1.7	635	79	68,220	1.9	35,900	63

<sup>a</sup> Mouse liver slices were divided into six equal samples for culturing. At the times indicated, medium in individual cultures was replaced for 30 min with medium containing 10  $\mu$ Ci of either [<sup>35</sup>S]methionine or [<sup>3</sup>H]uridine per ml as described previously (4). The slices were then washed and quick-frozen in phosphate-buffered saline. After thawing, they were dispersed in a polytron tissue homogenizer and assayed for incorporated label and protein content as described previously (4). In contrast to these slice cultures, monolayer cultures have been found to increase protein synthesis by six fold and RNA synthesis by threefold between 2 and 48 h in culture (4).

with 95% O<sub>2</sub>-5% CO<sub>2</sub> and gently shaken. For comparison, monolayers were also cultured under 95% O<sub>2</sub>.

Measurements of total macromolecular synthesis (Table 3) indicated that, unlike monolayer cultures (4), slice cultures maintain low total rates of RNA and protein synthesis which decline after 24 h. Furthermore, the slices gradually become fragmented in shaking cultures, so that after 24 h or so they may no longer maintain normal tissue structure or biochemical function and probably contain many dead or dying cells.

**Maintenance of liver-specific transcription in slices.** Since slice cultures could be maintained for 24 h with moderately active total protein and RNA synthesis, we have been able to compare the transcription rate of liver-specific and common mRNA sequences in nuclei from fresh liver with those from cells maintained in slices or in monolayer cultures for equivalent times. Autoradiographs from two such experiments are shown in Fig. 2 and 3, and the quantitation of the autoradiographic signals is given in Tables 4 and 5. A major general point can be made from these two experiments (and from a number of similar experiments not shown): liver-specific RNA transcription was maintained at a much higher rate in liver slices than in monolayers cultured under the same conditions. The rate of liver-specific transcription in

TABLE 4. Densitometric quantitation of transcription rate analysis

Probe	Relative density <sup>a</sup> (%)			Monolayer
	Slice, culture	Slice, no added hormones	Slice, no serum	
1	82	57	68	3
2	36	27	28	1
3	124	88	100	1
4	46	35	41	12
5	260	169	200	27
6	500	361	408	87
9	500	361	430	27
10	286	172	271	4
Al	22	19	23	0.2
Ac	197	234	158	187
bT	150	151	148	150
CHO-B	100	100	100	100

<sup>a</sup> Data from Fig. 2 were analyzed by densitometric scanning, along with data from a parallel experiment in which a sample of the same slice preparation was cultured in basal culture medium lacking serum. Signal for pBR322 on each filter was subtracted, and the different hybridization inputs were normalized by using the CHO-B signal as an internal standard (see Materials and Methods). Data for each transcription unit in the various cultures are presented in the table as percentages of the signal measured in fresh mouse liver (100%) for that transcription unit.

nuclei from slices usually averaged from 20 to 50% of that in liver nuclei for most genes and was approximately 5- to 20-fold higher than that in dispersed cells cultured in monolayers. In contrast, transcription of common mRNA sequences (actin,  $\alpha$ - and  $\beta$ -tubulin, CHO-B) was similar in slices, dispersed cultures, and fresh liver.

Although most liver-specific genes responded in a similar qualitative fashion to the different culture conditions, quantitative variations in response among the different genes and in different experiments were observed. For example, transcription of RNAs complementary to plivS-5, -6, and -9 actually appeared to increase in cultured liver slices compared with fresh liver in the first experiment (Fig. 2, Table 4). This effect was not observed in the second experiment (Fig. 3, Table 5). Apparent quantitative variations from experiment to experiment undoubtedly resulted in part from variations in the transcriptional profiles of the different fresh mouse livers used as controls in each experiment. Other variations may have arisen from subtle differences in the preparation and handling of the slices themselves. For example, slices cultured on a stationary platform (Fig. 3C) transcribed liver-specific genes at about twice the rate of slices suspended in shaking cultures (Fig. 3B, Table 5).

We also found that genes with the highest transcription rates in the normal liver (albumin, plivS-1, -2, and -3) appeared to decline the most in transcription in the monolayer cultures: typically, by 20- to perhaps more than 100-fold. Genes in this group were also more likely to show some decrease in transcription in cultured slices. The transcriptional signals for other liver-specific mRNA sequences (detected by plivS-4, -5, -9, and -10) typically declined by factors of 3 to 10 in monolayer cultures. The quantitative significance of the low residual signals measured in monolayer cultures is unclear, because although they are usually greater than the background hybridization to plasmid vector DNA alone, their statistical reliability is low. Also, without exploration of their source, for example, by hybridization of nascent RNA to a series of segments across a gene to demonstrate equimolar hybridization in each segment, it remains conceivable that the low signals are due to some type of cross-hybridization (39).

The only liver-specific RNA whose rate of synthesis was usually maintained at a normal or high rate in both slices and dispersed cultured cells is that complementary to plivS-6 (whose sequence is very similar to that of a rat transferrin cDNA; Citron, Grayson, Inouye, and Darnell, unpublished

TABLE 5. Densitometric quantitation of data in Fig. 3

Probe	Relative density <sup>a</sup> (%)		
	Shaking	Stationary	Monolayer
1	7	20	1
2	8	18	3
3	30	45	4
4	60	63	23
5	41	95	24
6	27	65	45
9	23	42	8
Al	13	25	2
Ac	28	53	46
aT	106	135	112
tR	33	24	64
28S	112	48	119

<sup>a</sup> Values for each transcription unit in the cultured tissue and cells are expressed as percentages of the signal measured in fresh sliced liver (100%).

results). A high level of transferrin mRNA transcription compared with other tissue-specific mRNAs has also been seen in cultured rat hepatoma cells (5).

A final observation concerning the data in Fig. 2 and Table 4 is that alterations in the culture medium had minimal effect on tissue-specific transcription in slices. Omission of supplemental insulin and hydrocortisone from the culture medium (Fig. 2C, Table 4) resulted in only a slight decrease in liver-specific transcription. Omission of serum from the medium resulted in a 50% decrease in total RNA synthesis, but had little or no effect on the relative pattern of tissue-specific transcription (Table 4).

**Is lost function in monolayers due to cell damage?** One possible explanation for loss of liver-specific transcriptional function in collagenase-dispersed cells is that the cells have been damaged somehow by the various proteolytic activities in the enzyme preparation. Therefore, a method for hepatocyte isolation was sought that did not employ proteolytic enzymes. Rat hepatocytes can be released from their normal tissue contacts by perfusion of the liver with EDTA (3). Similarly, we found that perfusion of mouse livers for 1 h with 2 to 10 mM EDTA in HEPES-buffered Hanks salts, followed by gentle teasing of the liver, resulted in the release of single cells (30 to 50% viable) from a large acellular mass, presumably the undigested reticular network and capsule. Viable cells were centrifuged through a Percoll cushion, and the cells (>95% viable) were plated and cultured as usual. Two hours after isolation, the nuclei of these cells were still active in transcription of most liver-specific genes (Fig. 4B), just as were cells isolated with collagenase (4). Just as with collagenase-dispersed cells, RNA complementary to plivS-1 (major urinary protein) was greatly decreased in EDTA-dispersed cells already after 2 h, and the transcription of actin was stimulated. After 24 h in culture on plastic culture dishes, the EDTA-dispersed cells (Fig. 4C) showed the same specific transcriptional decline as cells isolated with collagenase (Fig. 4D), suggesting that the action of collagenase was not the direct cause of the decline of tissue-specific transcription.

**Can tissue structure be reconstituted?** If disaggregated liver cells could reform a tissue structure in which tissue-specific transcription was now active, an experimental approach to discovering the cell or tissue components required for maximum tissue-specific transcription would be available. Although dispersed liver cells will form aggregates in suspension culture (17, 29), we have not found such aggregates to be stable or viable over the 24-h period required to observe

TABLE 7. Transcription in reengaged liver slice cultures compared with fresh sliced liver, normal slice cultures, and monolayer cultures

Probe	Relative density <sup>a</sup> (%)		
	Normal slice	Reengaged slice	Monolayer (EDTA)
1	12	5	<2
2	23	31	1
3	33	18	2
5	146	125	<41
6	98	73	48
9	36	18	<17
10	39	14	<10
Albumin	20	31	3
Actin	504	535	795
CHO-B	100	100	100
tRNA-arg	112	143	128

<sup>a</sup> Livers were disengaged by 1 h of perfusion with HEH (Fig. 4, Table 7) to prepare monolayer cultures, or were then perfused for an additional 1 h with basal culture medium (Table 7) to prepare reengaged slices. These cultures and standard slice preparations were exposed for a total of 24 h to basal culture medium under 5% CO<sub>2</sub> in O<sub>2</sub>. Transcription rate analysis was performed as described in the legend to Fig. 2 and footnote *a* of Table 4. Signals for each transcription unit are expressed as percentages of the signal in fresh slices of liver (100%). The "less than" symbol (<) indicates that the signal was no greater than the signal for nonspecific binding of labeled RNA to pBR322 DNA on the same filter.

the change in transcription of single cells. Perhaps the reticular network of fibers surrounding cells in intact liver (19) is necessary to mediate cell interactions or stabilize the tissue structure. We noticed when dispersing hepatocytes by EDTA perfusion (Fig. 4) that an acellular white mass (presumably the remnants of the intracellular matrix) remained after the cells were disengaged. It seemed possible that the hepatocytes might reengage within the liver structure after EDTA treatment if they were perfused with a complete tissue culture medium. Therefore, after a 1-h perfusion with the EDTA solution, perfusion was continued for another 30 to 80 min with the basal culture medium containing 20% fetal calf serum, a normal magnesium and calcium ion concentration, and no EDTA. To assay reengagement, livers were sliced or the capsule was stripped, and the released cells were counted (Table 6). At least 80% of hepatocytes were released from the liver after EDTA perfusion, whereas after another 50 min of perfusion with culture medium only 20% of the cells were released upon tissue slicing. Conversely, when livers were sliced after EDTA perfusion only acellular fragments were obtained, whereas substantial slices were produced from livers which had been additionally perfused with culture medium.

Although the slices from the reengaged liver were somewhat smaller and more easily fragmented than slices of normal liver, they survived the same 24-h culture period shaking in Erlenmeyer flasks. Nuclei isolated from these reengaged slices were found to transcribe liver-specific genes at rates similar to normal slices (Table 7) and well above the rate of transcription in nuclei from cells disengaged by EDTA but cultured as a monolayer.

## DISCUSSION

The experiments described in this and a previous paper (4) show that transcription of most liver-specific mRNA sequences, but not transcription of common mRNA sequences, declined sharply (10- to 100-fold) in cultured hepatocytes when they were disengaged from the normal tissue structure by either proteolytic or EDTA treatment. The composition of the culture medium and other alterations

TABLE 6. Cell release assay of reengagement<sup>a</sup>

Perfusate and treatment	No. of cells released × 10 <sup>6</sup>	% Viable
HEH for 60 min and tease	100	34
DMEM for 60 min and tease	2	0
HEH for 60 min and slice	80	24
HEH for 60 min, followed by BCM for 50 min and slice	20	<5

<sup>a</sup> Livers were perfused as indicated. Cells released into the medium by each treatment were counted in a hemocytometer and assayed for viability by trypan blue exclusion. Abbreviations: HEH, Hanks salt (see Materials and Methods), 10 mM EDTA, 5 mM HEPES (pH 7.3); DMEM, Dulbecco minimal essential medium; BCM, basal culture medium (containing serum and hormones). The flow rate in all cases was 6 ml/min, and otherwise perfusions were performed as described for the preparation of primary hepatocytes (see Materials and Methods).

of the culture environment had a minimal effect on maintaining tissue-specific transcription in isolated cells. However, transcription continued at high levels (20 to 100% of normal) when the cultured, explanted cells were maintained in a tissue-like organization. Added hormones and serum made slices perform only slightly better than slices without additives, emphasizing that tissue organization and not nutritional factors were most important for maintaining liver-specific transcription outside the body.

When the tissue was disengaged by EDTA and not proteolytic enzymes, the fibrous matrix between cells was maintained. When EDTA perfusion was stopped at a point where cells would have been dispersed upon rupture of the liver capsule, and perfusion was then continued with serum, the cells reengaged into a tissue organization. When slices of liver treated in this way were placed in culture, tissue-specific transcription was maintained. Cells in reengaged tissue were exposed to the same solutions and the same stresses as the cells separated by EDTA perfusion, with one exception: they quickly reestablished extracellular contacts in situ, and this seems to be crucial for maintaining the normal transcriptional profile. This experiment also implies that maximum tissue-specific gene transcription in the liver must rely on tissue organization, and inadequacies in the culture medium or cell damage are not the main cause of the immediate decline in specific transcription observed in dispersed hepatocytes. Of course it may be possible in the future, with a better understanding of the extracellular matrix, to reconstitute an environment that does support continued transcription in isolated dispersed cells, but this has not been successfully accomplished so far.

What role does this leave for hormones and other soluble factors in the maintenance of differentiated function in hepatocytes? First of all, we have not studied specifically those genes known to have a strong dependence on specific hormones (e.g., gluconeogenic enzymes [12]). We noted earlier (4) and also found in the present experiments that transcription of *plivS-1* (MUP) RNA was the most sensitive to disaggregation in our series of liver-specific genes. Several hormones, including both androgens and glucocorticoids, have a role in controlling this gene inside the animal (23), but the supply of both hormones does not maintain transcription of this gene in cultured cells. Even with this gene, however, we consistently obtained higher levels of transcription in slices than in cells. Also, our assay does not measure the possible hormonal requirements for longer-term maintenance of cellular integrity and function or for functions regulated at a posttranscriptional level (18).

Despite the dramatic drop in liver-specific transcription in isolated cells, very low but stable transcription of tissue-specific genes does appear to continue in hepatocytes outside the normal tissue organization. For instance, in the present experiments monolayer cells continued to transcribe liver-specific mRNAs at low but detectable rates (1 to 10% of normal liver) even after 10 days in culture (data not shown). Furthermore, in associated experiments (5) we find that hepatoma cell lines also transcribe many liver-specific genes, but at low levels similar to primary cultures. Some variant hepatoma cell lines, however, completely lack many liver-specific mRNAs and in several cases have ceased to transcribe some liver-specific mRNA sequences (5). Thus both primary cultures and hepatomas may have a low tissue-specific transcription rate in contrast to a very high level in liver cells in the proper tissue organization. We also note that the observed difference in hepatomas and liver cells is not just a reflection of growth rate, since regenerating liver

cells continue the liver-specific transcription pattern, (11) while regrowth of the liver occurs.

In this regard, it has been noted that a number of liver-specific mRNAs in rodents increase in concentration in the last quarter of fetal development (2, 32, 36), at a time when cell-cell contacts present in adult liver are formed (9, 31, 40, 46). Much of this increase appears to be due to faster transcription of already active genes during late stages of development (36). These measurements on fetal liver may reflect an increase in hepatocytes and a decrease in hematopoietic cells during development, but this change in cell composition is no more than a factor of 2 to 3 (35, 41), whereas the changes in mRNA and transcription approach or exceed a factor of 10. Thus it seems possible that already differentiated fetal hepatocytes as they mature do increase the frequency of transcription of liver-specific RNAs. Quantitative experiments with *in situ* hybridization and further analysis of transcription rates will be necessary to decide this point.

What sort of cellular control mechanisms related to a mature tissue structure might underlie an increased tissue-specific transcription rate? Either correct presentation and occupation of specific surface receptors or correct intracellular organization (perhaps maintained by the geometry of cell surface contacts) or both could be important in providing transcriptional signals. Our present experiments do not allow us to choose between these possibilities.

In either case, how might signals for differentiated function be communicated to tissue-specific genes in the nucleus and influence their transcription? Two distinct mechanisms can be imagined. First, specific soluble factors may be generated or modified upon receipt of a cell-surface signal or assumption of a critical intracellular structure. These factors might interact with specific sequences in the DNA or tissue-specific genes, or with other constituents of chromatin, to increase the activity of RNA polymerase II in transcribing these genes. The alternative, especially if maximum transcription depends on intracellular organization or cell shape, is that the nuclear structure itself might be important for specific patterns of transcription. Perhaps specific genes interact with a structure that provides increased access to RNA polymerase II, and that is disrupted when the cell is removed from the active tissue. Of course, even if genes have specific interaction sites in the nucleus diffusible factors might still play a role in specific transcription.

One final point concerning rates of tissue-specific transcription needs emphasis. At the present time, analyses for tissue-specific transcriptional function are popularly practiced by introducing plasmids or viruses bearing tissue-specific transcription units into partially differentiated and nondifferentiated cultured cells and comparing the amount of mRNA formed in the two cell types (24, 34, 37, 42, 44). Information on *trans*-acting tissue-specific transcriptional factors and DNA binding sites has already begun to accumulate as a result of those experiments. It will remain necessary, however, to find the basis not only of low or inefficient tissue-specific transcription, as is often observed in these experiments, but also for the robust transcriptional activities of fully differentiated cells.

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