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Liver-Specific RNA Metabolism in Hepatoma Cells: Variations in Transcription Rates and mRNA Levels

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The transcription rate and abundance of several liver-specific mRNAs as well as mRNAs common to many cell types were compared in a series of rodent hepatoma cell lines, normal liver cells, and primary hepatocyte cultures. The rat hepatoma cell line, Fao, which displays a liver-specific phenotype, contained eight of eight liver-specific mRNAs examined. However, the transcription rates of most liver-specific mRNAs were found to be low (1 to 30%) compared with normal liver in this and other differentiated cell lines. This low rate is similar to the transcription rates of liver-specific mRNA sequences measured in primary cultures of hepatocytes. Several variant cell lines that had lost differentiated traits contained few or none of the liver-specific mRNAs; clonal descendents which had regained differentiated function regained the tissue-specific mRNAs as a group, but at various concentrations. Because all of the changes observed in mRNA levels were not accompanied by parallel changes in transcription of the same sequences, differential posttranscriptional stabilization of the liver-specific mRNAs must also occur in the different cell lines. These results qualify the utility of cultured cell lines in the study of tissue-specific transcriptional control, but raise the possibility that posttranscriptional mechanisms act in cooperation with transcriptional controls to bring the level of tissue-specific mRNAs closer to those found in liver cells.

Cell lines derived from liver tumors have been widely used in attempts to study various processes that normally occur in hepatocytes, including the mechanisms of tissue-specific gene control. Particular hepatoma clones secrete or contain numerous liver-specific plasma proteins and enzymes and have been considered well differentiated (13). Studies using these clones and the tools of somatic cell genetics (12, 23, 34) and molecular genetics (4, 24, 25) have provided evidence that many liver functions are regulated in these cells in a concerted manner. For example, cell lines can be selected which no longer produce the two liver-specific enzymes central to the ability of normal hepatocytes to synthesize glucose (1, 2). Loss of the ability of variant cells grow without glucose is accompanied by the loss of other liver functions (e.g., serum albumin production) (23). When revertant cell lines are then selected for their ability to grow in glucose-free medium, the expression of many or all of these other liver functions is regained (12).

However, two important and general questions about these experiments, and cultured differentiated cell lines in general, remain unanswered. First, do immortalized, aneuploid, and culture-adapted cells exhibit patterns of gene control that resemble normal differentiated cells, or do they diverge in some potentially instructive way? For example, is the amount of a given liver-specific gene product in a well-differentiated hepatoma cell line quantitatively similar to that seen in the normal liver? How many separate liver functions are retained?

Second, when hepatoma clones sequentially lose and recover differentiated functions, at what level of regulation do these changes take place, and how are these changes related to the mechanisms in normal cells that control differentiation and tissue specificity?

To address these questions, we have measured the concentration and transcription rate of several liver-specific and common mRNAs in cell lines derived from hepatomas and compared these with normal liver and primary cultured hepatocytes.

MATERIALS AND METHODS

Cell lines and culture conditions. Mouse hepatoma clone BW1-J was derived from line BW1 (26). All other cell lines are clonal descendants of line H4IIEC3 (27) derived from the Reuber H35 hepatoma of rat (29); details concerning their isolation and properties are found in references cited below. All hepatoma cells were cultured in 10-cm Falcon petri dishes in modified (9) Ham F12 medium (15) supplemented with 5% fetal calf serum. Cultures were grown at 37°C in a humidified incubator flushed with 7% CO_2. Cells were detached for transfer and harvest with a saline solution containing 0.05% trypsin and 0.02% EDTA.

Primary cultures of mouse hepatocytes were prepared (6) by perfusion of mouse livers in situ with EDTA (10 mM) in modified Hank salts followed by collagenase (Worthington Diagnostics; class II) at 100 U/ml in Dulbecco minimal essential medium. Cells were cultured (7) in Dulbecco modified essential medium with 10% fetal calf serum, gentamycin (50 mg/liter), hydrocortisone (10 mg/liter), and insulin (10 mg/liter) in a humidified atmosphere of 5% CO_2 in air.

Recombinant plasmids. The plasmids used in these experiments are described in Table 1 and Fig. 4. Plasmids were grown in *Escherichia coli* strains, harvested, and purified as described previously (21).

Northern blot analysis of RNA concentration. RNA was isolated from the cultured hepatoma cells by lysis in guanidinium thiocyanate followed by centrifugation through cesium chloride (21). RNA was isolated from rat liver by homogenization in guanidinium thiocyanate followed by serial precipitations in guanidinium hydrochloride (5). RNA concentrations were determined from the optical density at

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TABLE 1. Recombinant cDNA-containing plasmids used in these experiments

Function	Abbreviation[a]	Encoded product	Source	Plasmid designation	Donor or reference
Liver functions	PH	Phenylalanine hydroxylase	Rat	prPH98	Robson et al. (30)
	PC	PepCk	Rat	pPCk2	Yoo-Warren et al. (35)
	Tf	Transferrin	Rat		J. Haggenvik
	Lg	Ligandin	Rat	pGST94	Kalinyak and Taylor (18)
	Albumin (Fig. 2)	Serum albumin	Rat	pR57	J. Sala-Trepat
	1	Major urinary protein	Mouse	plivS-1	Derman et al. (10)
	2	Unknown	Mouse	plivS-2	Derman et al. (10)
	3	α-1-Antitrypsin	Mouse	plivS-3	Derman et al. (10)
	4	Unknown	Mouse	plivS-4	Derman et al. (10)
	6	Transferrin or related	Mouse	plivS-6	Derman et al. (10)
	9	Unknown	Mouse	plivS-9	Derman et al. (10)
	10	Unknown	Mouse	plivS-10	Derman et al. (10)
	Al	Serum albumin	Mouse	pmalb2	Kioussis et al. (20)
Common functions and controls	p	(Plasmid vector)		pBR322	
	A	Unknown	Chinese hamster	CHO-A	Harpold et al. (16)
	B	Unknown	Chinese hamster	CHO-B	Harpold et al. (16)
	aT	α-Tubulin	Rat		P. Sharp
	bT	β-Tubulin	Human		N. Cowan
	Ac	β-Actin	Chicken	pA1	Cleveland et al. (8)
	tR	tRNA-arginine	*Xenopus laevis*	pyH48	D. Brown
	28S	28S rRNA	Mouse		N. Arnheim

[a] These abbreviations are used in the keys to Fig. 2 through 5. Plasmids containing albumin genomic sublcones are described in the legend to Fig. 3.

260 nm, and equal amounts of heat- and formamide-denatured total RNA were electrophoresed in formaldehyde-containing agarose gels, blotted to nitrocellulose filters, baked, and prehybridized; cDNA-containing plasmid DNAs were nick translated heat denatured, and hybridized to the filters in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 50 μg of yeast tRNA per ml, and 50 μg of heat-denatured salmon sperm DNA per ml in sealed plastic bags at 65°C for 18 h (6). Filters were washed in 2× SSC–0.2% sodium dodecyl sulfate at 65°C and then in 0.2× SSC–0.2% sodium dodecyl sulfate at 50°C.

Preparation of nuclei. Nuclei from the cell lines were prepared by suspending washed cell pellets at 4°C in a hypertonic buffer (60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris [ph 7.5], 0.1 mM phenylmethylsulfonyl fluoride, 0.4 M sucrose), adding Nonidet P-40 to 0.4% and diluting to 0.2% with the buffer, centrifuging the cells, suspending the cells in the hypertonic buffer, and homogenizing the cell pellet in a Dounce homogenizer. Nuclei from the primary cultured hepatocytes were prepared by Dounce homogenization in a hypotonic solution containing Triton X-100 as described previously (6). Nuclei from rat and mouse livers were purified by ultracentrifugation through a sucrose gradient after tissue homogenization (10). Washed nuclei were stored by quick freezing in liquid nitrogen in a buffer containing 40% glycerol, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, and 0.1 mM EDTA.

Preparation and hybridization of nascent-labeled nuclear RNA. Reaction in isolated nuclei, RNA extraction, and hybridization to dots of plasmid DNAs on nitrocellulose were carried out as described previously (7).

Autoradiography and densitometry. Preflashed Kodak X-AR1 film was exposed to washed, hybridized filters at −70°C with intensifying screens. After development, signal intensity was measured with a Helena Quick-Scan Jr. densitometer. The error of this procedure has been estimated to be less than ±30% for very low signals and ±4 to 11% for intense signals (7).

RESULTS

Characteristics of rat hepatoma-cell lines under study. A family of rat hepatoma cell lines (Fig. 1) originally descended from the Reuber H35 hepatoma (27, 29) has been established and studied extensively. Clone Fao, which expresses numerous liver functions (12, 13), is considered the parental cell type in most of these experiments. Clone C2 is a derivative of Fao in which liver functions including the ability to grow

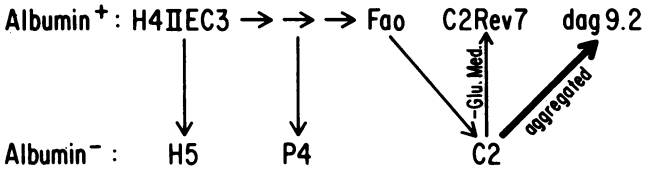

FIG. 1. Filiation of rat hepatoma cell lines used in these experiments. The cell lines on the bottom row (C2, H5, P4) fail to express several enzymes characteristic of liver cells and secrete no detectable serum albumin. The cell lines on the top row express some or all of these differentiated traits. The heavy arrow from C2 to *dag*9.2 indicates the high rate of production of *dag* cells after transient aggregation of C2 cells. Fuller discussion and references are presented in the text.

in glucose-free medium (12) were spontaneously lost. C2Rev7 is a revertant derived from C2 that was isolated by selection for cells which had recovered the ability to grow in glucose-free medium; other liver functions as well were expressed in C2Rev7 cells (12). *dag*9.2 cells were also derived from the C2 cell line: cells were allowed to aggregate transiently (11) and revertants designated *dag* were observed at high frequency. These cell lines manifest some liver-specific functions, but do not grow in the absence of glucose. Clones H5-6 and P4 are variants selected on the basis of altered morphology from the ancestral cell line. Neither of these latter two cloned lines expresses any liver functions examined, and neither gives rise to revertants to the differentiated phenotype at a detectable rate (12).

Specific mRNA concentrations in hepatocytes compared with various hepatoma cell lines. Recombinant plasmids are available that hybridize to various liver-specific and common RNAs (Table 1) and were used to measure the relative concentrations of different mRNAs in a variety of cell lines. Samples of equal amounts of RNA from each cell line were fractionated by size, transferred to nitrocellulose, and detected by hybridization to ^{32}P-labeled, cloned DNA (the Northern blot procedure [33]). For comparison, RNA from normal rat liver was included (Fig. 2). The liver-specific or liver-abundant mRNAs examined include those encoding three serum proteins produced by the liver (albumin, transferrin, and α-1-antitrypsin); phosphoenolpyruvate carboxykinase (PepCk), a principal enzyme in gluconeogenesis; two other liver-specific enzymes, phenylalanine hydroxylase and glutathione-*S*-transferase (ligandin); and two liver-specific mRNAs of unknown function (complementary to plivS-2 and plivS-4). Common mRNAs examined as controls included those encoding three cytoskeletal proteins (actin and α- and β-tubulin) and two mRNAs of unknown function that are found in many cell types (CHO-A and CHO-B).

The cell lines divide cleanly into two sets with respect to the accumulation of liver-specific mRNAs (Fig. 2, Table 2). Cell lines which express liver functions (Fao, dag 9.2, and C2Rev7) possessed all eight liver-specific mRNAs measured. Cell lines that lack liver functions (C2, H5, and P4) lacked all of the mRNAs with two exceptions: trace amounts of RNA hybridized to the ligandin probe, and a moderate amount of transferrin mRNA (about 5% as much as in normal liver) was detected in the samples from C2 and H5 cells. A specific correspondence between albumin protein synthesis and albumin mRNA concentration in these cells has been observed before (4).

The results showing that transferrin is maintained in all of the hepatoma cells are consistent with earlier experiments showing that mouse hepatocytes in primary culture lost 10 of 11 liver-specific mRNAs (6), but maintained the mRNA complementary to plivS-6, which has now been identified as the mouse equivalent of transferrin (B. Citron, R. Inouye, and J. E. Darnell, unpublished observations.) However, transferrin mRNA is a marker of hepatocyte differentiation, because kidney cells completely lack it and brain and spleen cells have only about 0.5% as much apparent transferrin mRNA as liver cells (28). Thus for both cultured hepatocytes and hepatoma cells transferrin seems to be one of the last tissue-specific markers to disappear.

Although Fao and the two revertant cell lines, *dag*9.2 and C2Rev7, contained each of eight liver-specific mRNAs, the relative amounts of the different mRNAs varied compared with those in liver cells. For example, phenylalanine hydroxylase was present in all cell lines in amounts equal to or greater than that found in liver. Albumin mRNA, however,

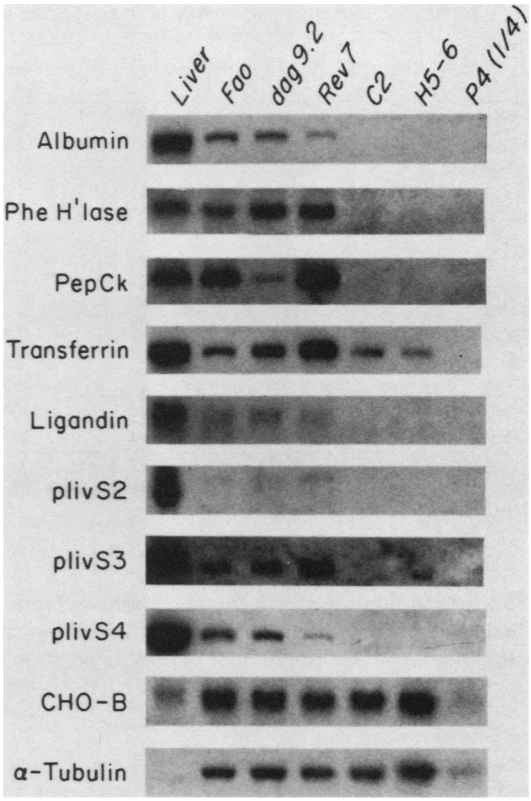

FIG. 2. Northern blot analysis of specific mRNA concentrations in rat hepatoma clones and liver. Total RNA was isolated from cultured cells and from one half of one rat liver (the other half was used to prepare nuclei for the experiment in Fig. 5) as described in Materials and Methods. A 25-μg sample of each RNA was analyzed by the Northern blot procedure (Materials and Methods). The integrity of the RNA and the equivalence of inputs were verified by observing the rRNA bands in the ethidium bromide-stained gels under UV irradiation. Four identical blots were prepared and were hybridized serially to three different probes; after each hybridization the probe was eluted by washing the blot in 0.1× SSC at 90°C for 30 min. The figure was prepared by collecting strips cut from autoradiographs of the blots; the probe used for each strip is indicated to the left. See Table 1 for a description of the plasmid probes used. Phe H'lase, Phenylalanine hydroxylase.

was present at from 5 to 16% of the concentration in liver. The lowest concentration of liver-specific mRNA in the hepatoma cells was that complementary to plivS-2, a liver-specific mRNA that is transcribed actively only in late fetal and adult hepatocytes (28).

Also, particular mRNAs varied in concentration among the three differentiated cell lines. In particular, PepCk was high in the two cell lines that will grow in glucose-free medium, Fao and C2Rev7. *dag*9.2 cells, which do not grow in glucose-free medium, contained about 10% as much PepCk mRNA.

Despite these variations, it is very clear that the loss and recovery of one liver-specific function (gluconeogenesis) was always accompanied by loss or recovery of many other liver-specific mRNAs. C2Rev7, the revertant of C2 selected in glucose-free medium, not only had more than twice as much PepCk mRNA as liver cells, but also contained significant amounts of phenylalanine hydroxylase, albumin, transferrin, ligandin, and α-1-antitrypsin mRNAs. Clearly these last four proteins are not required specifically for growth in glucose-free medium. The *dag* cells were selected only by a

TABLE 2. Relative abundance of liver-specific and common mRNAs in hepatoma clones compared with normal liver

Prepn	Relative abundance of mRNA[a] (%)					
	Fao	*dag*	C2Rev7	C2	H5	P4
Albumin	16	13	5	0	0	0
Phenylalanine hydroxylase	96	195	264	0	0	0
PepCk	114	17	235	0	0	0
Transferrin	9	20	50	6	3	<0.5
Ligandin	24	15	12	<1	<1	<0.5
plivS-2	0.5	0.5	1	0	0	0
plivS-3 (α-antitrypsin)	15	15	45	0	0	0
plivS-4	9	7	1	0	0	0
CHO-A	362	358	297	337	199	130
CHO-B	272	261	214	307	318	190
Actin	467	476	294	514	565	934
α-Tubulin	1,400	1,680	1,302	1,456	2,212	2,968
β-Tubulin	386	428	185	127	355	108

[a] Autoradiographs from the experiment shown in Fig. 2 were analyzed by densitometry (Materials and Methods). The value of each signal is expressed as a percentage of the corresponding signal in liver (100%). Analysis with the S1 nuclease protection assay (data not shown) confirmed the absence of albumin mRNA in C2, H5, and P4.

changed morphology and not by metabolic selection, but they had recovered all of the liver-specific mRNAs as well. These results strongly support the earlier suggestion (12) of the existence of some element(s) important for coordinate liver-specific gene control and indicate that this element(s) can be lost and regained.

Transcriptional and posttranscriptional regulation in the loss and recovery of albumin mRNA. To determine whether the observed variations in specific mRNA concentrations were the result of coordinated differences in the transcription rates of specific genes, we have assayed the transcription rates of a variety of genes as described in the accompanying paper (7). Nuclei were isolated from each of the cell lines and from rat liver, and RNA transcripts were elongated in the presence of radioactive UTP. Hybridization of equal amounts of radioactive RNA from the different samples to dots of excess plasmid DNA on nitrocellulose, followed by autoradiography and densitometry, affords an accurate measure of differential transcription on various genes at the time of nuclear isolation (7).

Our initial focus was on transcription of the albumin gene. Four cloned segments of this gene were available (31) and were used to obtain an accurate profile of polymerase activity across the entire albumin transcription unit (Fig. 3). In addition to these segments, we included a subclone immediately 5′ to the transcription initiation site and plasmids containing DNA complementary to β-tubulin mRNA and tRNA-arginine. These latter two DNA samples serve to verify that equivalent RNA inputs resulted in equivalent hybridization signals for two genes whose rate of transcription was expected to be similar in different cells. Fao and one of the revertants (C2Rev7) were compared with normal liver and with three undifferentiated variant cell lines (clone II5.0 is a variant that reverts at high frequency [23]).

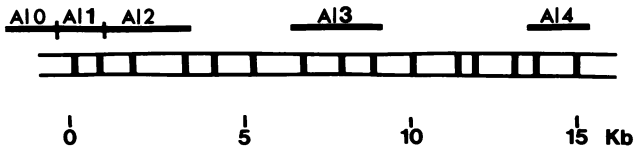

FIG. 3. Analysis of albumin gene transcription in rat hepatoma cells and normal liver. A map of the rat albumin gene is shown, with exons represented by vertical bars. The extent of each genomic subclone, a gift of J. Sala-Trepat, is indicated by horizontal strips above the gene. An alternative subclone nomenclature was used for clarity in this paper. The original, published names and approximate insert sizes of the subclones are as follows (31); JC (Al-0), 1 kilobase (kb); JB (Al-1), 1.2 kb; C (Al-2), 2.4 kb; B (Al-3), 2.6 kb; D (Al-4), 1.7-kb.

Two surprising results emerged from this experiment (Fig. 4). First, even in the well-differentiated clone, Fao, transcription across the albumin gene was much lower than in the normal liver. In this experiment the average signal intensity for the four genomic segments (Al-1, Al-2, Al-3, and Al-4) was estimated by densitometry to be about 25-fold lower in Fao cells relative to liver. This contrasts with a decrease of only about sixfold in the albumin mRNA concentration of Fao cells compared with that of liver cells (Fig. 2, Table 2). Little or no hybridization was obtained with the DNA upstream of the cap site (Al-0) in the transcription assay, and the relative distribution of polymerase activity among the four genomic segments was similar (and approximately equimolar) in nuclei from liver and Fao, indicating faithful copying of the entire transcription unit in the nuclei of both cell types. This decreased albumin transcription in the hepatoma cells was a differential event for polymerase II, because the β-tubulin signal was almost identical for the liver and hepatoma cell RNA samples.

The second surprising result was the barely detectable level of albumin gene transcription in the revertant, C2Rev7. Given the level of sensitivity of the transcription assay, we estimate that albumin transcription is reduced at least 1,000-fold in C2Rev7 compared with that of normal hepatocytes. Yet the albumin mRNA concentration is reduced only about 20-fold in this cell line (Table 2). The three variant cell lines which contained no detectable albumin mRNA (C2, P4, and II5.0) did not show transcription of the albumin gene at detectable levels. Thus, although loss of albumin mRNA in three variant cell lines apparently occurred at the level of transcription, increased conservation accounted for much of the recovery of the mRNA in at least one revertant.

Transcriptional analysis of other genes and another revertant cell line. To confirm and extend these results on the variable expression of the albumin gene, transcription assays were carried out with a broader sample of genes, including most of those whose relative mRNA concentrations were

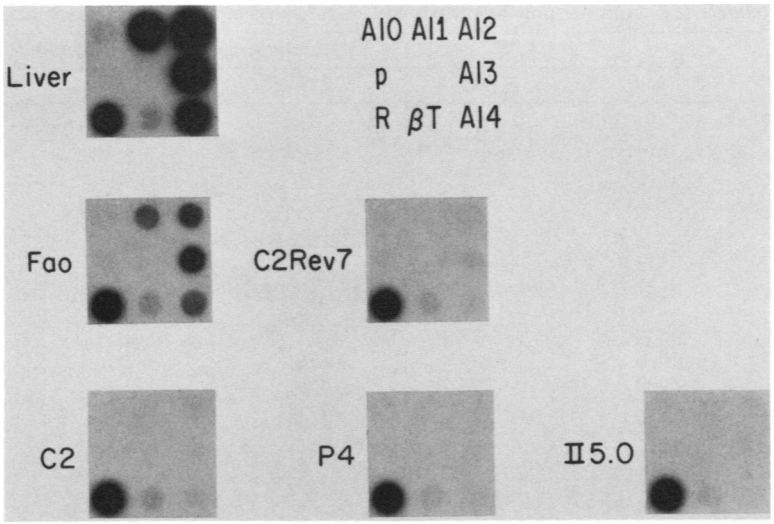

FIG. 4. Hybridization analysis of nascent-labeled nuclear RNA. Equal amounts of nascent-labeled nuclear RNA from cell lines and rat liver were prepared and hybridized to filters containing dots of various plasmid DNAs as described in Materials and Methods. The key indicates the arrangement of plasmid DNA dots on the replicate filters. Plasmids A10 through A14 are described in Fig. 3, and tR and bT are described in Table 1. Autoradiographs of filters after hybridization are presented.

determined earlier (Fig. 2, Table 2). Another revertant cell line, *dag*9.2, was also analyzed. In this experiment (Fig. 5, Table 3) the nuclei were taken from the same fresh rat hepatocytes and cultured cells as were used in the analysis of mRNA concentrations in Fig. 2. As with the albumin gene in the previous experiment (Fig. 3 and 4), disproportionately low transcription rates relative to mRNA concentrations were observed for the other liver-specific genes (PH, PepCk, transferrin, and ligand) in the differentiated cell lines (Fao, *dag*9.2, and C2Rev7). Nuclei from Fao cells, for example, transcribed these other liver-specific mRNAs from about 2.8 to about 7.8 times more slowly than would have been expected from the relative mRNA concentrations (Table 4). This indicates a greater stability for these sequences in the cultured cells compared with liver cells. In contrast, the ratio of mRNA concentration to transcription rate for three common mRNAs (actin, CHO-A, and CHO-B) was only slightly elevated or decreased when compared with that of liver cells. Tubulin mRNAs did seem to accumulate more in the cultured cells compared with their transcription rates, indicating increased posttranscriptional conservation of the tubulin mRNAs as well. Increased posttranscriptional conservation of the tubulin mRNAs has been observed before, both in cultured cells (17) and in comparisons of different tissues (28).

Although the two revertant cell lines, C2Rev7 and *dag*9.2, had recovered moderate levels of albumin mRNA (Fig. 2), the recovery was correlated with an increase in the albumin gene transcription only in *dag*9.2 cells (Fig. 5). In contrast, but consistent with the previous experiment (Fig. 4), C2Rev7 cells displayed little or no measurable increase in albumin gene transcription signal above the background level in the three non-albumin-producing cell lines (C2, H5-6, and P4).

Abortive RNA processing in variant cell lines. While the

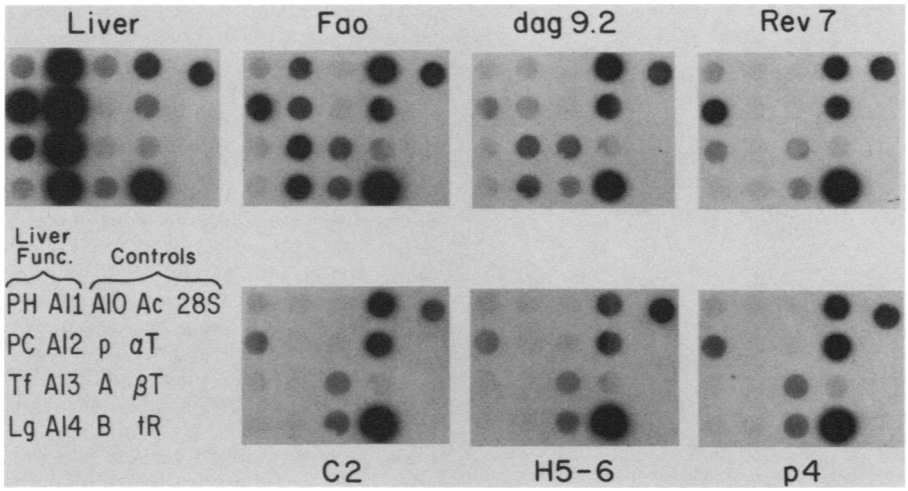

FIG. 5. Transcription rate analysis of various genes in rat hepatoma clones and liver. The experimental procedure is the same as in Fig. 4. Plasmids are described in Table 1 and the legend to Fig. 4.

TABLE 3. Relative transcription rates of various RNAs in hepatoma clones compared with normal liver

Probe	Relative transcription rate of RNA[a] (%)					
	Fao	*dag*	C2Rev7	C2	H5	P4
Albumin						
Al-1	2.8	0.6	0.03	<0.03	<0.03	<0.03
Al-2	2.8	0.8	<0.1	<0.1	<0.1	<0.1
Al-3	7.8	3.5	<0.2	<0.2	<0.2	<0.2
Al-4	6.1	2.9	0.06	<0.06	<0.06	<0.06
Combined data	4.4	1.8	<0.09	<0.09	<0.09	<0.09
Phenylalanine hydroxylase	33.8	51	106	38	21	30
PepCk	22.7	2.5	18.4	3.6	2.7	5.4
Transferrin	1.2	1.5	4.9	0.9	0.5	<0.5
Ligandin	<30	27	<14	19	<11	<11
CHO-A	1,400	1,246	616	980	644	1,148
CHO-B	200	98	114	190	134	166
β-Actin	262	254	141	220	66	157
α-Tubulin	283	223	258	266	201	552
β-Tubulin	136	78	231	101	83	148
tRNA-arginine	117	63	118	131	130	98
28S rRNA	100	72	88	47	138	87

[a] Densitometric quantitation (Materials and Methods) of data in Fig. 5 is presented. The value for each signal, after subtraction of the value for the pBR322 signal on the same filter (nonspecific background), is expressed as a percentage of the corresponding signal in rat liver (100%). Where a signal is no greater than the signal for pBR322 in the same hybridization, the "less than" symbol (<) is included.

differentiated hepatomas gave evidence of an increased stability of liver-specific transcripts, the undifferentiated variants (C2, H5-6, and P4) provided two apparent examples of continued transcription of liver-specific genes, although little or no mRNA accumulated in these cells. The mRNAs for PepCk and phenylalanine hydroxylase were undetectable in all of these variant cell lines (Fig. 2), yet substantial transcription signals for these mRNA sequences were obtained in each cell line (Fig. 5). For phenylalanine hydroxylase the transcription signals were essentially unchanged compared with the differentiated cells; for PepCk they were reduced 5- to 10-fold relative to Fao and C2Rev7, but were about the same as the transcriptional signal in the *dag*9.2 cells. These results imply a posttranscriptional failure either in RNA processing or mRNA stabilization in the undifferentiated cells. However, the present data are based on hybridization to a single cDNA clone for both PepCk and phenylalanine hydroxylase, and the possibility remains that the residual transcriptional signal generated in the dedifferentiated cells might not result from transcription of the entire phenylalanine hydroxylase or PepCk gene. It is even possible that some RNA other than phenylalanine hydroxylase or PepCk primary transcript could cross-hybridize to the clones. Neither of the cDNA clones has obvious high-level repeat sequences, but a lower-level repeat could conceivably give these results. To resolve this problem, an analysis with subclones across the gene (as was done for albumin, Fig. 3 and 4) is the best approach. Equimolar transcription across a set of subclones is strong evidence that a primary transcript is being accurately assessed.

These examples of an apparent positive posttranscriptional control of albumin in C2Rev7 and negative posttranscriptional control of PepCk and PH sequences in C2 cells can be contrasted to several examples of at least a limited degree of transcriptional regulation of liver-specific mRNAs for some genes in various cell lines. As noted above, the recovery of albumin mRNA in the revertant, *dag*9.2, to a level similar to Fao (Fig. 2) coincided with a proportionate recovery of albumin transcription (Tables 3 and 4). Also, phenylalanine hydroxylase mRNA was highest in C2Rev7, and this line also gave the highest transcription signal for this gene. PepCk mRNA is high in both Fao and in C2Rev7, and the transcription rate was high in both these two cell lines.

However, it is very clear that not every fluctuation in cytoplasmic mRNA concentration depended on a proportionate fluctuation in transcription rate of that mRNA sequence.

Transcription in a mouse hepatoma clone compared with mouse liver and cultured hepatocytes. In none of the rat hepatoma cells discussed so far, including the well-differentiated Fao parent, was the transcription rate of the tissue-specific genes comparable to normal liver (Fig. 4 and 5). This reduction might be a general property displayed by other hepatoma cell lines and perhaps by all cells cultured outside the normal tissue structure (7). Therefore a similar transcriptional analysis was made of the cloned mouse hepatoma line BW1-J (3), which expresses many liver functions. Normal mouse liver and primary mouse hepatocytes after 1 day in culture (6) were compared in the same assay, which included a panel of mouse liver-specific cDNA clones (10) and a mouse albumin cDNA clone (20).

TABLE 4. Ratios of abundance and transcription rate for selected mRNAs in hepatoma cell lines

Probe	Concentration/transcription[a]			
	Fao	C2	*dag*	C2Rev7
Albumin	3.6	0/0	7.2	>56
Phenylalanine hydroxylase	2.8	0/38	3.8	2.5
PepCk	5.0	0/18	6.8	12.8
Transferrin	7.8	6.7	13.3	10.2
CHO-A	0.26	0.34	0.29	0.48
CHO-B	1.4	1.6	2.7	1.9
Actin	1.8	2.3	1.9	2.1
α-Tubulin	4.9	5.5	7.5	5.0

[a] The data from Tables 2 and 3 are expressed as mRNA concentration divided by transcription rate. A number of greater than 1.0 (0.6 to 1.4) indicates the mRNA concentration has changed relative to liver more than the transcription rate has, and vice versa. Given the margin of error for densitometric analysis (Materials and Methods), these values should only be considered approximate (±40%).

The nuclei from BW1-J, like those from Fao, transcribed all tissue-specific mRNAs tested at a greatly reduced rate compared with that of normal mouse liver (Fig. 6 and Table 5). The transcription rate for most liver genes in BW1-J nuclei was not even as high as that in nuclei from hepatocytes after 24 h in primary culture, although the signals for albumin and transferrin mRNAs were clearly above background levels. The relatively high rate of albumin gene transcription in BW1-J nuclei (Table 5) compared with Fao nuclei (Table 3) is consistent with the relative albumin mRNA concentration in these two cell lines (32).

DISCUSSION

The hepatoma clones examined here are representative of cell lines which have been widely used as models for studying cell differentiation (19, 25). Fusion of these cell lines with nonproducing cells leads to the extinguishing of some liver cell functions (19, 22). Apparent mutations and reversions have also been described which lead to a coordinate change in the performance of multiple liver-specific functions (11, 12, 23). All of these results suggest underlying general control mechanisms for tissue-specific functions in liver-derived cells. Although liver-specific function in normal hepatocytes is controlled mainly at the level of transcription (10, 28), the basis for a liver-like phenotype had not been established for differentiated hepatomas, let alone mutants and revertants of the parental lines.

Our first priority in the current work, therefore, was to compare the production of liver-specific mRNAs in the differentiated hepatoma clones with that of normal liver cells. The results showed a broad array of liver-specific mRNAs to be present in the differentiated cells (Fao, *dag*9.2, C2Rev7) in concentrations ranging from 1% to more than 100% of those found in liver. These tissue-specific mRNAs encode a wide range of proteins, including three known serum proteins as well as intracellular enzymes involved in amino acid metabolism, gluconeogenesis, and detoxification reactions. In contrast, variant cell lines that were known to have lost some liver functions were found to have lost almost all of these liver-specific mRNAs. These findings indicate that the hepatoma clones serve as appropriate models for the study of the underlying concerted activation of tissue-specific genes.

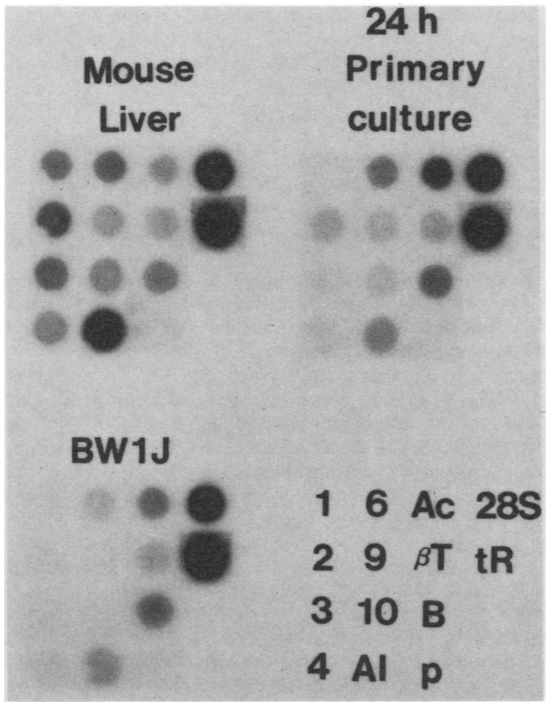

FIG. 6. Transcription rate analysis of various genes in mouse hepatoma clone BW1-J compared with that of mouse liver and primary cultured mouse hepatocytes. Primary cultures of mouse hepatocytes were prepared and cultured for 24 h as described in Materials and Methods. Liver-specific gene transcription rates in such cultures decline sharply in the first 24 h of culture and stabilize after 24 to 48 h (6). The same procedure as described in Fig. 4 was used to analyze nuclei from mouse liver, cultured BW1-J cells, and the primary hepatocytes. The plasmid DNAs used are indicated on the key and described in Table 1.

TABLE 5. Quantitation of data in Fig. 5

Probe	Relative density[a] (%)	
	Primary	BW1J
plivS-1	<6	<6
plivS-2	18	3
plivS-3	8	<4
plivS-4	6	6
plivS-6	71	20
plivS-9	57	4
plivS-10	26	<7
Albumin	13	14
Actin	388	176
β-Tubulin	115	105
CHO-B	147	150
28S rRNA	88	96
tRNA-arginine	75	108

[a] Densitometric analysis was applied as in Table 3.

However, in spite of the presence of many liver-specific mRNAs in the hepatoma cells at relatively high concentrations, the transcription rates of these mRNAs in the differentiated cultured cells were substantially lower than in cells in the liver. It should be noted that growing hepatocytes in regenerating liver continue to transcribe liver-specific mRNA sequences at the same rate as normal liver (14), so cell growth rate per se is not the dominant feature in determining transcription rates. In the accompanying paper (7), maximal transcription of tissue-specific mRNAs in mouse hepatocytes was linked to mature tissue structure, and a steady but much lower level of transcription of many different tissue-specific genes was found in hepatocytes removed from the normal tissue structure. Similar low levels of liver-specific transcription have also been observed in isolated rat hepatocytes (17). The low transcription rates of liver-specific genes in the cultured hepatoma cells is thus consistent with observations of normal cells in culture. However, there seems to be more conservation of liver-specific mRNAs in the cultured hepatoma cells than in true hepatocytes, since the hepatoma cells accumulate significantly higher amounts of these RNAs relative to their transcription rates (Table 4).

Our second goal has been to explore the molecular basis for the loss and recovery of differentiated traits in hepatoma cell variants. Comparison of the mRNA concentration data (Table 2) and the transcription rate data (Table 3) suggests that liver-specific genes may be regulated at both transcriptional and posttranscriptional levels in the variant cells that first lose and then recover liver-specific functions. This point is illustrated clearly in Table 4, where the ratio of mRNA

concentration to transcription rate was given for selected genes in the various cell lines. For example, the albumin gene is transcriptionally repressed in C2, and its reactivation in *dag*9.2 to a level equivalent to that in Fao appears to be transcriptionally mediated. However, the reappearance of albumin mRNA in C2Rev7, another revertant of C2, does not involve an equivalent recovery of transcription and so must involve significant increases in posttranscriptional stabilization or processing efficiency of the albumin mRNA. Rat hepatocytes in culture can also apparently achieve an increase in albumin mRNA based on an increased stability (17). It thus appears that different hepatoma clones may regulate the same gene differently.

Furthermore, within a single cell line the coordinated recovery of different liver-specific mRNAs may involve different levels of regulation. For example, despite the posttranscriptionally mediated recovery of albumin mRNA in C2Rev7, the return of PepCk mRNA and the increase of transferrin mRNA in C2Rev7 compared with that in C2 are both accompanied by transcriptional increases. Likewise, no single explanation is available for the recovery of the several liver-specific mRNAs in the *dag*9.2 cell line compared with its immediate parent, C2. Albumin transcription is increased, but PepCk, phenylalanine hydroxylase, and ligandin transcription are apparently not, yet all four mRNAs are accumulated at higher levels in *dag*9.2 cells than in C2 cells (Fig. 2, Table 2). In fact, the transcription rate of PepCk in *dag*9.2 cells is substantially less than that in C2 cells, yet *dag*9.2 cells have PepCk mRNA and C2 cells do not.

It thus appears as if these hepatoma cells are able to regulate a set of genes by coordinating both transcriptional and posttranscriptional mechanisms to yield a general phenotypic result—accumulation of many liver-specific mRNAs.

What are the implications of these results for models of tissue-specific gene control? At first glance, the apparent involvement of posttranscriptional gene activation and repression mechanisms in the regulation of at least three and possibly all of the five mRNAs examined in the rat cell lines would seem to contradict the previous observation that tissue-specific gene mRNA production in liver is controlled principally at the level of transcription (10). However, even the highest rates of transcription measured in the differentiated hepatoma cells are low and close to limits of detection in the first study of liver-specific mRNA production (10). With an improved autoradiographic assay (7), we can now monitor low rates of transcription reliably and find the described low levels of transcriptional activity in the hepatoma cells. Since these low levels are similar to those in primary cultures of hepatocytes, we suggest that the complete and concerted activation of tissue-specific genes in liver cells may actually require at least two steps: (i) the establishment of a basal level of mRNA synthesis and accumulation and (ii) an increase in polymerase II activity on specific genes that is somehow dependent on the assumption of the mature tissue structure. The first step might logically be expected to occur during embryonic induction and may be reflected in the low tissue-specific transcriptional output of cultured hepatocytes, cultured hepatoma cells, and possibly hepatocytes in the immature fetal liver (7, 28).

A complete understanding of the cellular mechanisms that activate and control tissue-specific genes will ultimately require isolation of the molecular machinery involved. The existence of procedures for selecting hepatoma clones that do (1, 2) or do not (23) display differentiated functions may allow a direct molecular genetic approach to this task. For example, the introduction of new genetic material under the control of constitutive promoters into a cell line like C2 may eventually lead to the isolation of genes that can complement the loss of function in these cells. The introduction of defined sequences into hepatoma cells (25) is already yielding information about critical regulatory sequences in the DNA of liver-specific genes. Other applications of these hepatoma clones to the problems of developmental gene regulation should emerge.

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