1985

Coordinated Control of Liver-Specific Genes in Cultured Cells From Mice and Rats

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COORDINATED CONTROL OF LIVER-SPECIFIC GENES IN CULTURED CELLS FROM MICE AND RATS

A thesis submitted to the Faculty
of The Rockefeller University
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

by

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February 1, 1985

The Rockefeller University
New York, New York

ABSTRACT

Assays of transcription rate and mRNA concentration were designed and employed to measure the production of a series of liver-specific and common mRNAs in fresh livers, primary hepatocyte cultures and hepatoma cell lines, derived from mice or rats. The transcription rates of many different liver-specific but no common mRNAs were found to decline sharply within 24 hours when hepatocytes from the liver were explanted and placed into primary culture. Hepatoma cells known to display liver-specific characteristics also transcribed liver-specific genes at low levels similar to primary cultured hepatocytes. In contrast, slices of liver tissue exposed to the same culture conditions for 24 hours maintained high rates of liver-specific transcription. These and other experiments demonstrate that: 1) maximum transcription of many liver-specific mRNAs depends on maintenance of the cells in a mature tissue structure; 2) low levels of liver-specific mRNA production can persist outside the tissue; and 3) the coordinate loss and recovery of many different liver functions observed in one line of hepatoma cell clones involves the regulation of tissue-specific mRNA concentrations through both transcriptional and post-transcriptional means.

ACKNOWLEDGEMENTS

With gratitude and admiration, I acknowledge both the challenge and support offered by my thesis adviser, James E. Darnell, Jr.

The following individuals played important and direct roles in particular aspects of the work described here: Allan Harrelson, Mary Weiss, Doug Jefferson and Lola Reid.

To the fifty or more members of the Darnell/Nevins lab who have populated the tenth floor ir the past five years: thank you all, for your immeasurable contributions, large and small.

To Dean Connelly and the many individuals throughout the Rockefeller community, who together have imparted to me a sense of what this wonderful place is all about: thank you all.

And special thanks to Cheryl, my wife, for patience, enthusiasm, "trust, love and support."

Portions of this thesis have been or will be published, as listed on the following page:

Clayton, D. F. and Darnell, J.E., Jr. (1983). Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. Mol. Cell Biol. 3, 1552-1561.

Clayton, D. F., Harrelson, A.L. and Darnell, J.E., Jr. (1985). Dependence of liver-specific transcription on tissue organization. submitted.

Clayton, D. F., Weiss, M.C. and Darnell, J.E., Jr. (1985). Liver-specific RNA metabolism in hepatoma cells: variations in transcription rates and mRNA levels. submitted.

Jefferson, D.M., Clayton, D.F., Darnell, J.E., Jr., and Reid, L. (1984). Posttranscriptional modulation of gene expression in cultured rat hepatocytes. Mol. Cell. Biol. 4, 1929-1934.

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CHAPTER ONE

INTRODUCTION

In the last few decades, the basic mechanisms that control a cell's character, from simple cells to the most complex, have been identified. Genes are now known to be made of DNA, and the flow of information from DNA into protein has been charted. That flow usually seems to be controlled at the level of mRNA production, and more specifically at the level of transcription (Darnell, 1982). The key enzymes involved in the process of transcription have been identified (Roeder, 1976), and much effort is now devoted to understanding how these enzymes and other regulatory factors interact with specific sequences in DNA to result in the controlled expression of single isolated genes (e.g., Ott et al., 1982, 1984; Schlissel and Brown, 1984; Bryan et al., 1983, and many others).

What we have yet even to glimpse, however, is this: how does a cell in a multicellular organism exert concerted control over the many different genes that collectively define it as a unique cell type? What mechanisms in a cell guide it in choosing which set of genes to transcribe? In short, why does a liver cell make liver-specific mRNA, and a brain cell not?

An ultimate answer to this question must address three

issues. First, the levels in the cell at which key regulatory decisions are made must be identified. For example, the final quantitative regulation of many gene products in mature tissues appears to be exerted through transcriptional controls (Darnell, 1982). But whether the underlying qualitative tissue-specific character of a developing cell first arises through transcriptional controls is unexamined.

Second, given knowledge of the biochemical level at which tissue specificity is regulated, the precise mechanisms of the controlling machinery must be understood. This may require biochemical or molecular genetic isolation of the machinery's components, and will undoubtedly depend on the fidelity and flexibility of the biological models available for study in the laboratory.

Finally, we must come to understand the historical process of development. How (and when) are the various controlling mechanisms in the various cell types activated and coordinated, to result in the right kind of cell, at the right spot, at the right time? Cell interactions undoubtedly play a significant role in this process of signalling and coordination, but the exact nature of these signals, and how they may lead to changes in general patterns of gene activity, are barely short of mysterious.

A general strategy for examining these questions would be to follow a single cell type, step-by-step through

development, monitoring the production of multiple tissue-specific products along the way. This would clearly be an ambitious undertaking. And since so few cells in the embryo participate in the earliest inductive processes, biochemical and molecular genetic analysis would be extremely difficult indeed.

An alternative approach would be to work backwards. Start with a mature tissue, ideally a large and simple tissue, and try to perturb it in such a way that large blocks of tissue-specific genes were simultaneously affected. If such perturbations could be defined, one could then apply biochemical and molecular genetic techniques to understand and/or isolate the mechanisms involved in coordinate control of tissue-specific genes. With this knowledge, one might then be in a position to approach the embryological issues directly.

In my thesis work, I have taken this second approach to studying the control of tissue-specificity, using the rodent liver as a biological subject. The liver is an especially good source of a large number of well-differentiated cells of a highly homogeneous type. Furthermore, the uniformity of liver tissue structure makes it relatively easy to observe the function of liver cells in their normal environment, and to manipulate that environment.

I have exploited both physical and genetic techniques to

perturb the function of liver cells. Using the tools of molecular genetics to monitor the production of tissue-specific mRNAs in liver cells in various states, I have come to three broad conclusions: first, that specialized mechanisms do in fact seem to exist that coordinate the production of many different mRNAs related only in their tissue-specific pattern of expression; second, that one of these mechanisms results in a high level of transcription of tissue specific genes as long as the cells remain a part of a mature, intact tissue; and third, that another mechanism may exist to control or maintain the low level of tissue-specific mRNA production observed in fetal liver cells or in mature cells outside the liver tissue.

KEY ATTRIBUTES OF THE RODENT LIVER

The liver has numerous advantages as a subject for the analysis of tissue-specific gene control, including: large size, cellular homogeneity, uniform architecture, biochemical specialization, simple developmental history, established techniques for primary culture, and related clonal cell lines that maintain some tissue specific functions in culture. Because of these advantages, cDNA-containing plasmids have already been isolated that hybridize only to liver messenger RNA (Derman, et al., 1981), and basic aspects of mRNA metabolism in liver cells have already been investigated (e.g., Powell, et al., 1984; Friedman, et al., 1984; Barth, et

al. 1982; Muglia and Locker, 1984; Derman, et al., 1981).

LARGE SIZE

After the skin, the liver is the largest organ in the body (Jones and Spring-Mills, 1983). In adult mammals, the liver accounts for 1% or more of total body weight (Doljanski, 1960). The liver of a single 30 gram mouse, for example, weighs about 1 gram, and yields more than 100 million cells when the tissue is disaggregated (e.g., Clayton and Darnell, 1983).

CELLULAR HOMOGENEITY

A single cell type, the hepatocyte, constitutes about 90% of the mass of the mouse liver (Greengard et al., 1972). Though the hepatocyte may only account for about half of the number of cells in the liver, many hepatocytes contain at least two nuclei, so about 75% of the transcriptional machinery of the liver is contained in hepatocytes.

In addition to hepatocytes, the liver is also known to contain endothelial cells, Kuppfer cells, fat-storing ("Ito") cells, and cells of the biliary tree (e.g., Jones and Spring-Mills, 1983).

UNIFORM ARCHITECTURE

Unlike many other differentiated tissues, the architecture of the liver is more or less uniform throughout

the organ. Hepatocytes, linked by tight junctions, desmosomes and other connections (Montesano et al., 1975; Evans, 1980; Gallin et al., 1983) and contacting an extracellular matrix (Reid and Jefferson, 1984), are arranged in plates separated by sinusoids (frequently fenestrated capillaries) that are lined by rather scarce endothelial cells (Jones and Spring-Mills, 1983). These plates are arranged like spokes in a wheel to result in the fundamental functional unit, the liver lobule, that is repeated thousands of times throughout the tissue. The plates radiate from the "central vein" outward to the perimeter of the lobule, which is defined by (usually) five or six "portal triads" each consisting of a bile ductule and the terminating branches of the portal vein and hepatic artery. Blood enters the liver through the portal vein and hepatic artery, and exits each lobule through the central vein to the inferior vena cava. Bile, secreted by hepatocytes into junctional spaces called "bile canaliculi," flows in the opposite direction, and ultimately exits the lobule through the bile ductules at the portal triads.

Each liver lobule is on the order of 1 mm in diameter; hepatocytes themselves are typically 20-40 microns in diameter (Jones and Spring-Mills, 1983). Thus the radius of each lobule, that is the length of the hepatocytic plate, is about 10-20 cells. Some differences in cellular function have been noted across the lobule (Jungerman and Katz, 1982; Gebhardt and Mecke, 1983), apparently because of environmental differences: cells at the portal perimeter receive blood rich

in oxygen and nutrients (and toxic products), and cells toward the center receive the blood only after the portal cells.

The surface of the hepatocyte contains three structurally and functionally distinct domains (Evans, 1980; Cook et al., 1983): an inter-hepatocytic junction, the bile cannalicular membrane, and the side facing the sinusoidal space. Junctions critical for tissue structure and function are maintained between the hepatocytes, and extracellular material is most prominent along the sinusoidal face (Jones and Spring-Mills, 1983). Constituents of the extracellular material include collagens (Types I, III, IV and V), laminins, proteoglycans (e.g., heparan sulfate,), and fibronectin (Rojkind et al., 1980; Diegelman et al., 1983; Yamada, 1983; Reid and Jefferson, 1984).

BIOCHEMICAL SPECIALIZATION

Probably more cell-specific products (intracellular enzymes and secreted proteins) are known for hepatocytes than any other cell type (Jungerman and Katz, 1982). Tissue-specific functions of hepatocytes include: production of serum proteins (e.g., albumin, transferrin, complement, blood coagulation factors, lipoproteins); production of bile and urinary proteins (e.g., major urinary protein of mice); conjugation reactions (e.g., glutathione-S-transferase or ligandin); gluconeogenesis (e.g., phosphoenolpyruvate carboxykinase); amino acid metabolism (e.g., tyrosine amino transferase, phenylalanine hydroxylase); blood clearance

functions (e.g., asialoglycoprotein receptor, Ashwell and Harford, 1982), and storage of fats and glycogen.

DEVELOPMENTAL HISTORY

Production of hepatocytes in the embryo appears to require a specific interaction between cells in one region of the endoderm and cells in one region of the mesoderm (Houssaint, 1980; Le Douarin, 1975; Fukado-Taira, 1981). This interaction probably requires actual contact between cell membranes (Le Douarin, 1975). In the mouse, hepatocytic cells migrate out from the endoderm into the mesoderm around the ninth day of development (Theiler, 1972); this interaction between endodermally-derived cells (hepatocytes) and mesodermally-derived cells (e.g., endothelial cells) appears to persist throughout the life of the organ.

The exact lineage of each cell type in the liver has been examined (Shiojiri, 1981) but remains disputed. Some groups suggest the continued existence in the adult organ of a stem cell that can produce biliary cells as well as hepatocytes (Grisham, 1979). Similarly, the actual number of steps or stages involved in the production of hepatocytes is not well defined. Examinations of cells early in the formation of the liver, for example, often rely on the hepatocyte's ability to produce alpha-fetoprotein (Houssaint, 1980; Shiojiri, 1981). Although production of this protein is essentially restricted to the liver in normal adults, this and other "liver-specific" proteins like serum albumin may be produced at other locations

in the embryo (Dziadek and Andrews, 1983).

There is little evidence, however, for a complex series of inductions or stages in the development of hepatocytes, as is seen with tissues like the blood or the brain. Hepatocytes appear to assume their fundamental qualitative characteristics shortly after tissue induction. The quantitative aspects of tissue-specific function may be amplified as development proceeds (see Chapter 7), and hormone- or nutrition-dependent liver-specific functions (Greengard, 1969; 1975) may not be prominent until the appropriate environment exists. Also, differences in isozyme patterns have been observed for liver cells in the fetus compared to cells in the adult liver (Guguen-Guillouzo and Guillouzo, 1983). Critical changes in the hormonal and nutritional environment occur shortly before birth and at weaning (Greengard, 1969, 1975).

One other aspect of the developing liver should be noted: the liver is the primary site of hematopoiesis in the fetus. Developing blood cells first colonize the liver around the twelfth day of gestation in the mouse (Houssaint, 1980) and constitute about 50% of the number of cells in the liver through the rest of fetal development, with hepatocytes constituting about 30% of the total cell number until hematopoietic colonies decrease after birth (Silini et al., 1967; Paul et al., 1969).

PRIMARY CULTURE

To isolate cells from the intact tissue, the junctions between hepatocytes and between hepatocytes and the extracellular matrix must be broken. Techniques for doing this efficiently with rat liver evolved in the late 1960's and early 1970's (Howard et al., 1967; Berry and Friend, 1969) and culminated in the simple two-step perfusion scheme of Seglen (1976). Livers were briefly perfused with an EDTA solution to chelate the divalent cations responsible for tight junctions and other contacts, and then perfused with a solution of collagenase to digest the extracellular matrix. Low-speed centrifugation of the resulting cell suspension was found to yield a population highly enriched in hepatocytes, which are considerably larger than other non-parenchymal cells. Similar approaches were also found to work with mouse livers (Klaunig, et al., 1981a).

For subsequent culture of isolated hepatocytes, a number of media and substrates have been described (for reviews, see Grisham, 1979; Pitot and Sirica, 1980; Harris and Cornell, 1983). Typically, insulin, hydrocortisone and fetal calf serum are added to Eagle's medium or a subsequent modification (Dulbecco's, Williams, Ham's). Serum-free media have recently been devised (Enat, et al., 1984). Cells may be plated on ordinary tissue-culture plastic, on collagen substrates or floating gels (e.g., Michalopoulous and Pitot, 1975), on more complex extracellular matrix preparations (Rojkind, et al., 1980), or in some cases they may be maintained in suspension

(Jeejeebhoy, 1976).

Regardless of the method of cell preparation and culture, however, the general observation has been that cultured hepatocytes suffer a quantitative decrease in tissue-specific function after a few days (Pitot and Sirica, 1980). Functions known to decline include albumin production, glycogen synthesis, some enzymes of the urea cycle, synthesis and secretion of alpha-2-mu-globulin, cytochrome P-450 inducibility, and tyrosine amino transferase inducibility (Pitot and Sirica, 1980; Michaloupoulous and Pitot, 1975; Newman and Guzelian, 1982).

Also, cultured hepatocytes have a very low rate of cell division, despite the fact that cells in the liver are capable of dividing rapidly at least once or twice to regenerate lost tissue (Pitot and Sirica, 1980). At least two groups have reported culture media and techniques that are supposed to result in sustained cell division in cultured hepatocytes (Leffert et al., 1977; Enat, et al., 1984), but these results have been controversial. Some techniques have been reported which are supposed to enhance other tissue-specific functions in cultured hepatocytes (e.g., Michalopoulous and Pitot, 1975; Rojkind, et al., 1980). The mechanisms by which these enhancements occur have not been examined, and in few if any cases has it been possible to compare the level of improved function to the level of function seen in the normal cell in its normal environment. Work described later in this thesis

will address some of these points.

HEPATOMA CELL LINES

In addition to perturbing cells physically by altering their environment, one might hope to perturb cells genetically through the techniques of somatic cell genetics. However, these techniques require cells that can be grown in culture for long periods of time. As described above, normal hepatocytes do not seem to have this capability.

However, clonal cell lines have been isolated from hepatomas that do grow in culture, and in some cases some level of liver-specific function has been found to be retained (Pitot and Sirica, 1980, Deschatrette and Weiss, 1974). Working with such cell lines, Mary Weiss and her colleagues have produced evidence for underlying coordinate control of multiple tissue-specific functions in these cultured cells. 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 (Bertolotti, 1977a; 1977b). Loss of the ability to grow without glucose is accompanied by the loss of other liver functions (e.g., serum albumin production) in these variant cell lines (Moore and Weiss, 1982). When "revertant" cell lines are then selected for their ability to grow in glucose-free medium, many or all of these other liver functions are regained (Deschatrette et al., 1980). Other experiments using cell hybridization techniques (reviewed in Weiss, 1982) have shown that when

"differentiated" hepatoma cell lines are fused with "non-differentiated" cell lines, some liver-specific functions are "extinguished," suggesting the existence of diffusable factors involved in the coordinate control of liver-specific genes expression (Mevel-Ninio and Weiss, 1981; Weiss, 1982; Killary and Fournier, 1984).

However, a quantitative comparison of liver function in these cultured cells and in normal liver cells has never been made. Nor has the biochemical level of control, much less the molecular mechanisms involved, been established for the observed changes in function. An investigation of these issues is presented in Chapter 6.

mRNA METABOLISM

The experimental advantages of the liver as a subject for a molecular analysis of tissue-specific gene control were recognized by Derman et al. (1981), who isolated a series of eleven distinct cDNA-containing plasmids which hybridized to liver RNA but not to RNA from brain or a cultured cell line. They considered these plasmids, and their complementary mRNAs, "liver-specific," therefore. A later analysis by Powell et al. (1984) found that these same mRNAs were also greatly reduced in concentration in the spleen, the kidney and several other tissues as well.

To establish the biochemical level of control over these eleven liver-specific mRNAs, Derman et al. (1981) employed an

assay of instantaneous transcription rate to compare transcription of these mRNAs in liver and in brain. The assay involved elongation of nascent transcripts in isolated nuclei from the two tissues in the presence of alpha-^{32}P-labeled UTP. The amount of UTP incorporated into each RNA should be proportional to its relative rate of transcription at the time of nuclear isolation. By hybridizing the labeled nuclear RNA to excess amounts of the various plasmid DNA probes (including control plasmids that hybridized to "common" mRNAs with a wide tissue distribution) followed by scintillation counting, Derman et al. were able to obtain a quantitative value for the differential transcription rate of the mRNAS complementary to the various cDNA probes in liver and in brain. (A control experiment compared this approach with a pulse label of intact cells, scoring for production of several common mRNAs, and found the two techniques to be in agreement. There are several technical advantages to an assay based on isolated nuclei instead of intact cells, not the least of which is the ability to measure transcription rates in fresh, intact tissues.)

The result was that brain and cultured cell nuclei were found to transcribe all eleven liver-specific mRNAs at rates at least 50- to 100-fold below the rates found in liver nuclei. Derman et al. concluded that quantitative regulation over these liver-specific mRNAs must reside principally at the level of transcription, since post-transcriptional levels (processing or cytoplasmic stability) did not need to be invoked to explain the large differences in mRNA concentration

in the three tissues.

These results were replicated and extended in the study by Powell, et al. (1984). Again, the transcription of liver-specific genes was found to be low in brain, and in kidney and spleen as well.

Using the same panel of liver-specific cDNA probes, Friedman, et al. (1984) found no evidence for significant changes in transcription rate or abundance of most liver-specific mRNAs in the regenerating liver. However, substantial changes in mRNAs for cytoskeletal elements (actin and tubulin) were observed, apparently mediated largely at a post-transcriptional level. Other examples of post-transcriptionally-mediated changes in these mRNAs in liver cells were described in Powell, et al. (1984), and in work presented in this thesis.

General changes in liver-specific mRNA metabolism in the developing liver have been examined by Powell, et al. (1984) and by Barth, et al. (1982). The general conclusion has been that most liver-specific mRNAs increase in concentration from low to moderate levels to near-adult levels during the last few days of development. Some particular mRNAs appear to be virtually absent in the liver before birth. These results are consistent with observations of protein and enzyme levels in the fetal liver, discussed earlier. Additionally, Powell et al. (1984) measured the transcription as well as concentration of liver-specific genes in the developing liver, and found a

general correspondence between transcription rate and concentration for various mRNAs. However, in two cases (mRNAs complementary to plivS-7 and plivS-8) the mRNAs were absent from the fetal liver despite a transcriptional signal equivalent to the adult, raising the possibility that these mRNAs might be regulated in development at a post-transcriptional level.

In addition to these investigations of coordinate control of liver-specific mRNAs, a number of laboratories have focused on individual liver-specific mRNAs, including those encoding albumin (Kioussis et al., 1981; Sargent, et al., 1981; Muglia and Locker, 1984), alpha-fetoprotein (Kioussis et al., 1981; Tilghman and Belayew, 1982), phenylalanine hydroxylase (Robson, et al., 1982), tyrosine amino transferase (Granner and Hargrove, 1983), tryptophan oxygenase (Schmid et al., 1982), major urinary protein (Knopf, et al, 1984; Shahan and Derman, 1984); phosphoenolpyruvate carboxykinase (Yoo-Warren, 1983) glutathione-S-transferase (Kalinyak and Taylor, 1982), antithrombin III (Stackhouse et al., 1982), complement proteins (Carrol and Porter, 1982), and several others (Costanzo et al., 1983).

WHAT MAKES LIVER MAKE LIVER-SPECIFIC mRNA?

The fundamental question addressed by this thesis (above) can be broken into two components, based on the information

reviewed so far:

- what makes adult liver cells transcribe liver-specific mRNAs at a high rate?
- what makes fetal liver cells begin to make liver-specific mRNA in the first place?

The majority of this thesis will address the first question. The first issue that must be established is whether adult liver cells transcribe liver-specific mRNAs at a high rate because of some internal, autonomous and permanent modification (for example, modification of DNA), or whether transcription depends on some external signal or set of conditions (for example, appropriate hormones or cell-cell contacts). This issue is resolved in this thesis work by examining liver-specific mRNA production in primary cultures of normal hepatocytes.

The second question (why does a liver cell begin to make liver-specific mRNA in the first place?) is more difficult to explore, for the reasons given in the first few paragraphs. However, interesting parallels exist between the behavior of fetal liver cells and certain cultured hepatoma cell lines. In this work, the behavior of these cell lines is explored at a molecular level (using cloned cDNAs), and inferences are drawn about the possible molecular mechanisms of control in the normal developing liver cell.

The author hopes that this work may contribute in some way to an eventual understanding of the processes of cell

differentiation and tissue-specific gene control.

CHAPTER TWO

MATERIALS AND METHODS

BACTERIAL STRAINS, CULTURES AND PLASMIDS

The following bacterial strains and techniques used for manipulating recombinant cDNA-containing plasmids are all fully described in Maniatis et al. (1980). Bacterial strains used for preparation of plasmid DNAs included HB101, RR1, and C600. Strains were stored at -20°C or -70°C in LB medium containing 15-50% glycerol. Bacteria were cultured in M9 or LB medium, and transformed with plasmid DNAs by the calcium chloride procedure, followed by selection on agar plates containing appropriate antibiotics. Plasmid DNAs were prepared from single colonies or frozen stocks, following amplification with chloramphenicol, typically by alkaline lysis. Plasmid DNAs were purified by centrifugation through cesium chloride gradients in the presence of ethidium bromide. Integrity and identity of plasmid DNAs were monitored by digesting samples with appropriate restriction enzymes to release cDNA inserts, followed by electrophoresis through 1-1.5% agarose gels in .04 M Tris-acetate, .002 M EDTA, containing ethidium bromide for detection under ultraviolet illumination; cDNA insert sizes were estimated by comparison with the digestion patterns of known standards. Concentrations of plasmid DNAs and protein contaminants were estimated by measuring the absorbance at 260 nm and 280 nm,

respectively. DNA samples were typically stored under refrigeration in 10 mM Tris (pH 7.4), 1 mM EDTA.

Plasmid DNAs used in these experiments are described in Tables 1 and 2.

ISOLATION OF HEPATOCYTES

Adult male C57/B6 mice (Charles River Breeding Laboratories, Inc.) were injected intraperitoneally with an 8:1 mixture of nembutal (50 mg/ml, Abbott Laboratories) and heparin (1000 U/ml, Riker Laboratories). After an alcohol wash, a midline ventral incision was made with sterile scissors, and a sterile Teflon cannula was inserted through the right ventricle into the inferior vena cava. The portal vein was cut as perfusion was begun with a sterile wash solution (HEH) of Hank's salts, EDTA, and Hepes buffer at 37°C [HEH (per liter): 400 mg KCl, 60 mg KH_2PO_4, 8000 mg NaCl, 90 mg $Na_2HPO_4-7H_2O$, 1000 mg dextrose, 17 mg phenol red, 10 mM EDTA, 5 mM Hepes (pH 7.3)]. A ligature around the inferior vena cava below the liver was tightened, and the flow rate was increased to ca. 7 ml/min and continued for a total of 4 min. The HEH wash solution was then replaced by a prewarmed and filtered solution of collagenase (100 units/ml, Class II, Worthington Diagnostics) in Dulbecco's modified essential medium with glucose at 4,500 mg/liter (DMEM) (GIBCO Laboratories). Perfusion was continued for about 7 min or until 50 ml of the collagenase solution had been used. This procedure was carried out under a 40-W lamp, and the liver

was maintained at about 30°C. Perfused livers were cut out, rinsed in warm DMEM and gently agitated with forceps to release cells. Cells were counted and tested for viability by staining with 0.45% trypan blue in phosphate-buffered saline or DMEM. Typically, $>10^8$ total cells were obtained in the original suspension, and 60-80% of these were large, nonstaining hepatocytic cells.

Cells were released if the initial perfusion with HEH was omitted, but with lower viablity and yield (about 40% viability of 7×10^7 cells). Where appropriate (Chapter 4, Table 6) other constituents (insulin, hydrocortisone, testosterone, cycloheximide, concanavalin A, demecolcine, cytochalasin B) were added at the concentrations used in cell culture to the perfusate or to the initial cell suspension.

Hepatocytes were released from the liver without using collagenase by perfusion typically for one hour with the HEH solution described above. Flow rate was maintained at 7 ml/min with a peristaltic pump and temperature at 37°C, and a mixture of 95% O_2 and 5% CO_2 was bubbled slowly through the perfusate reservoir. After dispersion and centrifugation, the cells from one liver were resuspended in the perfusing solution (12 ml) followed by addition of 13.7 ml iso-osmotic Percoll (Pharmacia) (made by adding 1 part 1.5 M NaCl to 9 parts Percoll), to result in a solution density of 1.07 g/ml. The suspension was centrifuged at room temperature for 30 min. (1500 xg) to yield a pellet of approximately 3×10^7

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 one hour, with several changes.

CULTURE OF HEPATOCYTES

Cell suspensions were centrifuged for 3 min at 50 x g to pellet the hepatocytes. Cells were suspended in an appropriate volume of culture medium (below) and plated on 150 mm (Falcon) or 100 mm (Nunc) plastic tissue culture dishes or (as indicated in Table 6) on bacterial petri dishes covered with biomatrix substrate (Enat, et al., 1984; Reid et al., 1984) that were prepared and provided by Lola Reid and Doug Jefferson. Cells were routinely plated at a density of $6x10^4$ / cm^2. This density was found to be nearly saturating for the attachment of viable cells and resulted in the attachment of few dead cells. The medium was changed at 2 hours with a single rinse, allowing for the removal of dead and unattached cells (viable cells attached within this time). Under standard culture conditions, cells in 150 mm plates were maintained under 12-15 ml of medium, with changes of medium every 1-2 days. Cultures were maintained in humidified incubators (5% CO_2 in air) or under a humidified atmosphere of 5% CO_2 in O_2, at 37°C. For suspension cultures, hepatocytes were placed in Erlenmeyer flasks on a rotary shaker (50-100 rpm) at 37°C under an atmosphere of 5% CO_2,

95% O_2.

HEPATOCYTE CULTURE MEDIA AND ADDITIVES

For standard or "basal" culture conditions cells were cultured in Dulbecco's minimal essential medium with insulin ("Iletin I," Eli Lilly) and hydrocortisone sodium succinate ("Solu-Cortef," Upjohn) added at 10 mg/L each, heat-inactivated fetal bovine serum (Sterile Systems) added to 10%, and gentamicin (Schering) added to 50 mg/L. In early experiments, inosine (GIBCO Laboratories) at 10 mg/L and 0.2% bovine serum albumin (Miles Laboratories, Inc.) were also added, and penicillin and streptomycin (100 U/liter) were used instead of gentamicin, but these were found to have no detectable effect on the cultures. Non-heat-inactivated serum also had no different effect on cultures or transcription.

Alterations of the standard medium described in Chapter 4 involved the following components: horse serum (Sterile Systems) at 10%, testosterone (Sigma) at 10^{-6} - 10^{-7} M, epidermal growth factor (Sigma) at 2 mg/L, glucagon (Sigma) at 10 mg/L, all-trans retinoic acid (Eastman) at 10^{-7} M, Dimethyl sulfoxide (DMSO, Sigma) at 1.5%, dibutyryl cyclic AMP (Sigma) at 10^{-4} - 10^{-3} M, sodium butyrate at 5 mM, cycloheximide (Sigma) at 22 mg/L, concanavalin A (Calbiochem) at 25 mg/L, CaCl2 at 6 mM, A23187 (Calbiochem) at 1 mg/L with DMSO at 1.5%, sodium ortho-vanadate at 10 mcM, cytochalasin B (Sigma) at 2 x 10^{-5} M plus 0.5% DMSO, demecolcine (Sigma)

at 1 mcM. The hormonally defined medium of Enat et al. (1984) was provided by Lola Reid and Doug Jefferson, and contains the following: RPMI medium with 100 U/ml penicillin, 100 mcg/ml streptomycin, insulin (265 mU/ml), glucagon (10 mcg/ml), epidermal growth factor (50 mcg/ml), prolactin (2 mU/ml), human growth hormone (10 mcU/ml), linoleic acid (5 mcg/ml with delipidated BSA, 1 mcg/ml), copper (10^{-7} M), selenium (3×10^{-10} M), and zinc (5×10^{-11} M). Arginine-free ornithine supplemented "basal medium" of Leffert et al. (1977) and Williams D medium were obtained from Gibco and were supplemented as described for basal medium above; arginine-free medium was not changed after cell initial cell attachment and rinsing (Leffert, et al., 1977). Hepatocytes were infected with Adenovirus 5 by addition to the medium of 5000 particles/cell after 36 hours in culture (this resulted in cell death about 2 days later, and transcription of the E1A gene was robust by 6 hours post-infection). Liver extract was prepared by polytron homogenization in the cold of adult mouse livers in Dulbecco's minimal essential medium and clarification by centrifugation (5 min at 1000 xg); supernatants were prepared from a total of 6 livers in 3 installments over 24 hours, and added immediately to the culture medium (36 ml) of 3×10^7 hepatocytes (about 1/3 of one liver).

CO-CULTURE OF HEPATOCYTES WITH OTHER CELL TYPES

Rat liver epithelial cells were provided by C. Guguen-

Guillouzo and co-cultured with mouse hepatocytes as described (Guguen-Guillouzo, et al., 1983). Bovine aortic endothelial cells were provided by Eric Jaffe and seeded at a 1:1 ratio onto attached hepatocytes after 2 h in culture. Mouse liver non-parenchymal cells were centrifuged at a high speed (500xg for 10 min.) from the supernatant after freshly dissociated hepatocytes had been pelleted at low speed, yielding about 3 x 10^7 non-parenchymal cells from 1 mouse liver; 1-10 times as many non-parenchymal 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's modified essential medium or with basal culture medium (above). Liver lobes were arranged flat side down on the chopping platform of the McIlwain Tissue Chopper (Brinkman), and were cut twice at right angles. Although a slicing thickness of 0.325 mm was usually used, thickness could be varied between 0.150-0.450 mm with negligible consistent effect on specific gene transcription. Reengaged livers (preparation described in text) 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 equivalent were placed in two 500 ml Erlenmeyer flasks with 70-100 ml each. A

constant stream of the humidified gas mixture was introduced into the flasks throughout the culture period. The rotary shaking rate was adjusted (usually 70-80 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 was avoided). Medium was changed first at 2 hours and typically every 4-8 hours thereafter, by gravity sedimentation in the culture flasks and aspiration of the supernatant.

HEPATOMA CELL LINES AND CULTURE CONDITIONS

Mouse hepatoma clone BW1-J was derived from line BW1 (Peterson and Weiss, 1972). All other cell lines are clonal descendants of line H4IIEC3 (Pitot et al., 1964) derived from the Reuber H35 hepatoma of rat (Reuber, 1961); details concerning their isolation and properties are found in references cited in Chapter 6. Cultures grown in the laboratory of M. Weiss were cultured in 10 cm Falcon petri dishes in modified (Coon and Weiss, 1969) Ham's F12 medium (Ham, 1965) supplemented with 5% fetal calf serum and 100 U/liter each penicillin and streptomycin, under 7% CO_2 at 37°C. Cells grown by the author were cultured in 10 or 15 cm Falcon tissue culture dishes under 5% CO_2 in a similar medium: Ham's F12 medium supplemented with 0.5X MEM Amino Acids (GIBCO Laboratories), 5% fetal calf serum and gentamicin (50 mg/liter). No significant differences in RNA profiles were observed in cells cultured either way. Cells

were detached for transfer and harvest with a saline solution containing 0.05% trypsin and 0.02% EDTA (GIBCO Laboratories).

METABOLIC LABELING OF RNA AND PROTEIN IN INTACT CELLS

For assay of protein synthesis rate, the medium from 10 cm monolayer cultures or slice cultures in 50 ml Erlenmeyer flasks was replaced with a small volume of methionine-free medium (Joklik modification; GIBCO Laboratories) supplemented with 10 mcCi/ml of ^{35}S-methionine (New England Nuclear Corp.) and the additives described for standard culture medium. After 30 min., cells were washed several times then collected in ice-cold phosphate-buffered saline and sonicated briefly; protein was precipitated from samples in 10% trichloroacetic acid, collected, washed on glass fiber filters, and counted in scintillation fluid. RNA labeling was similar, except that ^{3}H-uridine (New England Nuclear Corp) was dried under vacuum and added to the standard culture medium at 10 mcCi/ml. RNA was bound to DE81 filters (Whatman), washed several times in 5% dibasic sodium phosphate-20 mM sodium pyrophosphate, rinsed, dried and counted in scintillant. Total protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories).

To label proteins in cells for two-dimensional electrophoresis, ^{35}S-methionine was added to methionine-free medium (above) at 100-200 mcCi/ml; cells were washed and lysed in solution A of O'Farrell (1975). Livers in mice were labeled by intraperitoneal injection of 1-2 mCi of ^{35}S-

methionine.

To label RNA in cells for filter hybridization analysis of specific mRNAs, ^{3}H-uridine was added to standard culture medium at 400 mcCi/ml.

POLYACRYLAMIDE GEL ANALYSIS OF PROTEINS IN CELL EXTRACTS

The E2 protein of Ad-5 was detected in acetone-precipitated sonic extracts of ^{35}S-methione labelled hepatocytes 6-12 hours after infection, by immunoblotting (Feldman et al., 1982).

Non-equilibrium two-dimensional gel electrophoresis (isoelectric focusing range: pH 3-10; 10% acrylamide running gel in second dimension) of labelled proteins from livers and hepatocyte cultures were performed as described (O'Farrell, et al., 1977; O'Farrell, 1975).

ISOLATION OF RNA FROM CELLS AND TISSUES

In experiments in Chapter 3, RNA was isolated from whole liver polytron homogenates and scraped cultured cells by phenol extraction. In Chapter 6, RNA was isolated from whole rat livers by the guanidinium/precipitation method, and from cultured cells by the guanidinium/cesium chloride method.

PHENOL EXTRACTION: Cells and tissues were homogenized in a buffer of 50 mM sodium acetate - 10 mM EDTA - 0.5 % sodium dodecyl sulfate (pH 5.1) plus two volumes of phenol-

chloroform - isoamyl alcohol (12:12:1). After 3-5 extractions with phenol-chloroform-isoamyl alcohol at 65°C, RNA was precipitated at -20°C by addition of 0.2 M NaCl and 2 volumes ethanol.

GUANIDINIUM/PRECIPITATION METHOD (Chirgwin, et al., 1979): Livers were polytron homogenized in a buffer made by mixing and dissolving (per 99 ml) 5 ml 1.0 M lithium citrate, 60 g guanidinium thiocyanate (Fluka), 0.7 ml 2-mercaptoethanol, followed by adding 1 ml 10% lithium dodecyl sulfate (BDH) and filtering. Approximately 10 ml of this solution were used per gram of tissue. Homogenates were mixed with 0.05 volumes 1M acetic acid and 1 volume 95% ethanol, chilled at -20°C and centrifuged 30 minutes at 5000 rpm in a Beckman JS13 rotor at -5 to 0°C. Pellets were resuspended in one half the previous volume, in a filtered solution of 7.5 M guanidinium hydrochloride (Schwarz Mann), 25 mM lithium citrate and 1 mM dithiothreotol; 0.025 volumes acetic acid and 0.5 volumes ethanol were added, followed by chilling at -20°C and centrifugation. After several cycles of resuspension and precipitation, the pellet was washed in 95% ethanol, extracted with chloroform-4% isoamyl alcohol, and ethanol precipitated.

GUANIDINIUM/CESIUM CHLORIDE METHOD: The exact procedure is described in detail in Maniatis et al. (1982). Briefly, cells were resuspended in 5 volumes of 6M guanidinium isothiocyanate, 5 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, and 0.5% Sarkosyl. 1 gram cesium chloride per 2.5 ml homogenate was added, and the homogenate was

layered onto a 5.7 M CsCl cushion, followed by ultracentrifugation to pellet the RNA. After organic extraction, the RNA was ethanol precipitated.

For preparation of poly(A)+ RNA, selection on oligodeoxythymidylate columns (Collaborative Research) was used, as described in Maniatis et al., 1982.

RNA concentrations were estimated by measuring optical density at 260 nm.

NORTHERN BLOT ANALYSIS OF RNA CONCENTRATION

Equal amounts of RNA samples to be compared were denatured at 65°C, 5 minutes, in 50% formamide, 6% formaldehyde, 1x BE buffer (20x is 0.4 M borate, pH 8.3 and 20 mM EDTA), 5% glycerol, with trace amounts of bromphenol blue and xylene cyanol included as markers for electrophoresis. Samples were loaded onto horizontal agarose gels containing: 1x BE buffer, 1.5% agarose (Seakem), 2.8% formaldehyde (16.6 ml 37% formaldehyde added to 200 ml), and 0.5 mg/L ethidium bromide for uv detection of ribosomal RNA when total RNAs were used. After electrophoresis in 1x BE and 1.85% formaldehyde (100-300 volts, until bromphenol blue reached the bottom of the gel), gels were placed under nitrocellulose that had been soaked in 20 X SSC (1x is 0.15 M NaCl plus 0.015 M sodium citrate) in a "Southern blot" arrangement (Southern, 1975). After transfer overnight, blots were baked for 2-4 hours at 80°C in a vacuum oven. Blots were

pre-hybridized in sealed plastic bags in a solution of 5x SSC, 1x Denhardt's solution (50x, per liter: 10 g Ficoll, 10 g polyvinylpyrolidone, 10 g bovine serum albumin), 50 mcg/ml yeast tRNA and 50 mcg/ml denatured (100°C) salmon-sperm DNA, for 2 hours at 65°C. Plasmid DNAs were nick-translated (Rigby, et al. 1977; Maniatis et al., 1982) using ^{32}P-dCTP (New England Nuclear Corp.) to a specific activity of at least 10^8 cpm/mcg and purified by ethanol precipitation; plasmid DNAs were denatured at 100°C, and about 500,000 cpm/ml was added to a small volume of the hybridization solution, otherwise the same as the prehybridization solution. After hybridization for 18 h at 65°C with shaking, filters were washed in 2x SSC plus 0.2% SDS at 65°C, then in 0.2x SSC plus 0.2% SDS at 50°C, and then autoradiographed. In some cases filters were re-hybridized to a second probe after washing for 30 min in 0.1x SSC at 90°C to remove the previous probe.

DOT HYBRID ASSAY OF SPECIFIC MRNA TRANSCRIPTION RATES

To measure the rate of transcription of specific genes in different populations of cells, I isolated nuclei from the cells and allowed previously initiated RNA to elongate in these nuclei in the presence of radiolabelled UTP (details given below). Under the conditions used, the amount of radiolabelled UTP incorporated into RNA is proportional to the number of polymerases active on each primary transcription unit at the time of nuclear isolation (Darnell,

1982). In experiments conducted by myself (not shown) and others (e.g., Derman et al., 1981; reviewed in Darnell, 1982), this method of measuring transcription rate has been found to give similar results to methods based on pulse label of intact cells.

Equal amounts of radiolabelled RNA from the different samples of nuclei were hybridized to dots of liver-specific and common cDNA-containing plasmid DNAs, followed by autoradiographic detection and densitometric quantitation of RNAse-resistant hybrids. The relative amount of transcriptional activity devoted to each liver-specific gene could thus be compared, both between different samples and to the relatively constant transcription of the common genes.

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 radioactive signal intensity and exposure times. The experiment shown in Figure 1 and Table 3 shows that both of these conditions are met for the procedures I have used. Two different amounts of nascent-labelled mouse nuclear RNA were hybridized to filters containing plasmid dots (Tables 1 and 2) representing the full range of signal intensities encountered in these

experiments. Decreasing the amount of RNA normally used in a hybridization by a factor of about five (4.73) resulted in an equivalent decrease in signal intensity for all the plasmids (Table 3). Thus, the hybridization signal varied linearly with the amount of RNA in solution, across a range of concentrations. 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 (Figure 1B). The weakest signal that could be read with confidence at the lowest exposure (Figure 1A) was about 1/20 of the strongest and since the strongest signal increased proportionately with a fivefold increase in exposure time (Figure 1C) I was able to obtain a linear measure of signal intensities over a range of at least a hundred-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, see Table 3). For densitometric analysis of very low signals, therefore, exposures were always used in which the signal for pBR322, representing the background of non-specific hybridization, was clearly detectable. As shown in Table 3, errors of $\pm$4-11% were estimated in measurements of moderately to intensely transcribed genes (plivS 1-4 and 9, albumin, tRNA) and errors of up to $\pm$30% for genes giving weak transcriptional signals (actin, alpha-tubulin).

Table 1: Plasmids Encoding Liver-Specific mRNAs.

Abbreviation	Encoded Product	Source	Designation	Donor or Ref.
1	major urinary protein	mouse	plivS-1	Derman, et al., 1981
2	unknown	mouse	plivS-2	"
3	a-1-antitrypsin	mouse	plivS-3	"
4	unknown	mouse	plivS-4	"
5	unknown	mouse	plivS-5	"
6	transferrin or related	mouse	plivS-6	"
7	unknown	mouse	plivS-7	"
8	unknown	mouse	plivS-8	"
9	unknown	mouse	plivS-9	"
10	unknown	mouse	plivS-10	"
11	unknown	mouse	plivS-10	"
Al	serum albumin	mouse	pmalb2	Kioussis, et al., 1981
aFP	alpha-feto-protein	mouse	paFP2	"
Al	serum albumin	rat	pR57	J. Sala-Trepat
PH	phenylalanine hydroxylase	rat	prPH98	Robson et al. 1982
PC	PepCk	rat	pPCk2	Yoo-Warren et al., 1983
Tf	transferrin	rat	--	J. Jaggenvik
Lg	ligandin	rat	pGST94	Kalinyak and Taylor, 1982
A11, A12 A13, A14	albumin genomic subclones	rat	JB, C B and D	Sargent et al, 1981

Abbreviations as used in keys to figures. Encoded products of plivS-3 and plivS-6 were recently identified (Darnell et al., manuscripts in preparation).

Table 2: Plasmids Encoding Common RNAs and Control Plasmids.

Abbreviation	Encoded Product	Source	Designation	Donor or Ref.
p	(vector)	--	pBR322	--
AL0	(genomic sub-clone upstream from albumin)	rat	JC	Sargent et al., 1981
Ac	beta-actin	chicken	pA1	Cleveland, et al., 1981
aT	alpha-tubulin	rat	--	P. Sharp
bT	beta-tubulin	rat	--	N. Cowan
A	unknown	Chinese Hamster	pCHO-A	Harpold et al., 1979
B	"	"	pCHO-B	"
C	"	"	pCHO-C	"
E	"	"	pCHO-E	"
F	"	"	pCHO-F	"
tR	tRNA-arginine	X. laevis	pyH48	D. Brown
tM	tRNA-methionine	"	pXatmet1	"
28S	28S rRNA	mouse	--	N. Arnheim

Abbreviations as used in keys to figures.

Figure 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-labelled RNA from mouse liver nuclei prepared by tissue slicing and Dounce homogenization (Materials and Methods). Autoradiographs of the filters following hybridization and washing are shown. Two hybridizations were performed with different inputs of the same labelled RNA sample, and two exposures of the filters to pre-flashed X-ray film were taken to result in the four images shown. A and C: 2.5×10^6 cpm of labelled nuclear RNA was hybridized to the filter. B and D: 11.8×10^6 cpm of labelled nuclear RNA (equivalent to that isolated from 2/3 of one liver) was hybridized to the filter. The "1x" exposure (A and B) was for 3.8 hours, and the "5x" (4.73) exposure (C and D) was for 18 hours. The key shows the arrangement of plasmid DNA dots on the filters. (Table 3 gives quantitation of these autoradiographic signals).

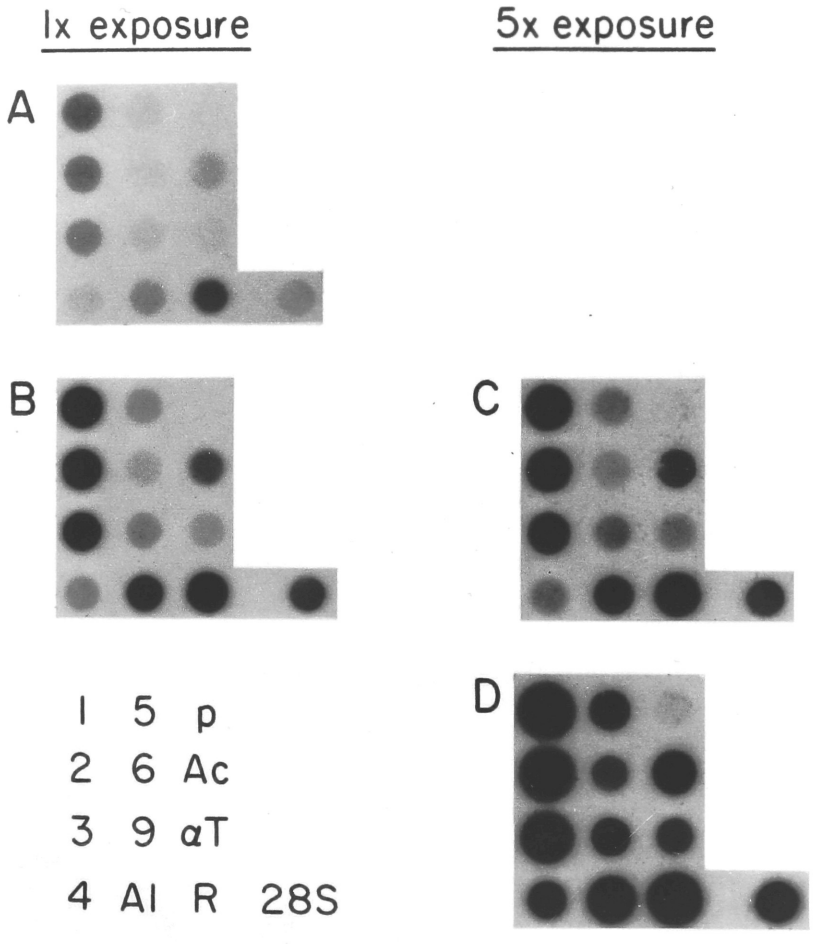

1x exposure
5x exposure
A
B
C
D
1 5 p
2 6 Ac
3 9 αT
4 Al R 28S

Table 3: Densitometric Analysis of Autoradiographs from Transcription Experiments (Figure 1).

	Hi input Lo exposure	Lo input Hi exposure	Lo input Lo exposure x 4.73	Avg.	max. error (±%)
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
Al	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		

The densities of dots on autoradiographs were determined by optical scanning and integration. The numerical values for the low input, low exposure combination (Figure 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 on the high input, high exposure combination (Figure 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.

The detailed protocol for the dot hybrid analysis of transcription rate using isolated nuclei is given below.

ISOLATION OF NUCLEI FROM CELLS AND TISSUES

Three general methods were used to isolate nuclei from cells and tissue for transcription rate analysis. The first method involved Dounce homogenization in a hypotonic buffer and was used for cultured cells, cultured slices, and slices of fresh liver (following perfusion with about 20 ml PBS or DMEM): after cells or tissue slices were washed several times in ice cold PBS or DMEM, they were washed in cold RSB (10 mM NaCl, 10 mM Tris (pH 7.4), 3.5 mM $MgCl_2$, 14 mM 2-mercaptoethanol) and swollen for several minutes in RSB (about 10 volumes per tissue volume) to which Triton X-100 was added to 0.12% (for cells) or 1.8 - 0.2 % (for slices) . After adding KCl to 150 mM, the tissue was homogenized in a Dounce homogenizer (B pestle) with 5-8 strokes (for slices) or 10-20 strokes (for isolated cells). Nuclei were pelleted by centrifugation (3 min at 800 xg).

The second method (Derman et al, 1981) was used occasionally with fresh livers, and resulted in cleaner preparations of nuclei although this had no detectable effect on the results of the transcription assay. One rinsed liver was minced and homogenized in 10 ml [0.32 M sucrose, 5 mM Hepes (pH 6.9), 1 mM DTT, 1 mM $MgCl_2$] with a motor driven Teflon pestle (about 1000 rpm). After centrifugation (5 min.

at 1000 xg) the nuclear pellet was resuspended in 11 ml [2.1 M sucrose, 5 mM Hepes (pH 6.9), 1 mM DTT, 1 mM $MgCl_2$], layered onto 1.5 ml of the resuspension buffer and centrifuged in an SW-40 rotor for one hour at 20,000 rpm, 4°C. The pellet of nuclei was washed in 0.25 M sucrose, 5 mM Hepes (pH 6.9), 1 mM DTT, 1 mM $MgCl_2$.

The third was used in some experiments with the rat hepatoma cells. Nuclei were prepared by resuspending washed cell pellets in a hypertonic buffer [60 mM KCl, 15 mM NaCl, 1mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris (pH7.5), 0.1 mM PMSF, 0.4 M sucrose] adding NP-40 to 0.4% and diluting to 0.2% with the buffer, centrifuging the cells, resuspending in the hypertonic buffer, and homogenizing the cell pellet in a Dounce homogenizer (B pestle).

Washed nuclei were stored in some cases by quick freezing in a buffer containing 40% glycerol, 50 mM Tris (pH 8.0) 5 mM $MgCl_2$, and 0.1 mM EDTA.

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 $MgCl_2$, 1mM $MnCl_2$, and 14 mM 2-mercaptoethanol. They were resuspended and incubated for 12 minutes at 30°C in 1-3 volumes of the same buffer (pH 7.9) but with the addition of (α-^{32}P) UTP (NEN 007H) to 1 mCi/ml, phosphocreatine to 10 mM, and creatine kinase to 100 ug/ml. Nuclei were lysed by

diluting the reaction (typically 0.3-1.0 ml) with 1.5 ml of a high salt buffer (HSB) (0.5 M NaCl, 0.01 M Tris (pH 7.4), 0.05 M $MgCl_2$, 0.002 M $CaCl_2$) and pipetting. 200-400 units of RNase-free DNase (Worthington) was added and the reaction held at room temperature with pipetting for about 1 minute. SDS (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 8.4, 0.5% SDS. After extracting with phenol:chloroform:isoamyl alcohol (24:24:1) and chloroform, RNA was precipitated twice from a large volume (6-8 ml) at -20°C with yeast tRNA carrier (10 mcg/ml), 0.2 M NaCl, and 2.5 volumes ethanol.

Incorporated radioactivity was measured by scintillation counting after spotting aliquots on DE81 paper, and washing several times (5 minutes each) first in a solution of dibasic sodium phosphate (50 g/L) and sodium pyrophosphate (8.9 g/L), then in dibasic sodium phosphate (50 g/L) alone, then briefly once each in distilled water and 95% ethanol. Incorporation of approximately 1.0 (+/- 0.5) cpm/nucleus was usually achieved with nuclei from mouse liver and slice cultures, and higher amounts with nuclei from monolayer cultures.

HYBRIDIZATION OF NUCLEAR RNA TO PLASMID DNA

Recombinant plasmid DNAs were denatured by boiling for 8 min in 0.1 N NaoH, 10 mM Tris, 1 mM EDTA. They were quick-cooled on ice and diluted 15-fold with cold 2M NaCl (10^{-5} g/liter ethidium bromide was sometimes included as a marker

for filter preparation). Using a 96-well "dot blot" apparatus (Schleicher and Schuell) the DNAs were applied to nitrocellulose, pre-wet with 6xSSC. After washing 2-3 times with 6xSSC, the filters were baked (Kafatos et al, 1979; Thomas, 1980). A dot of plasmid vector DNA (pBR322) was always included as a measure of non-specific hybridization. Filters were prehybridized for two hours at 65°C in a solution containing 2x TESS [50 mM TES, 300 mM NaCl, 10 mM EDTA, 0.2% SDS; pH to 7.4] plus 1 mg/ml yeast tRNA, 0.2% Ficoll, 0.2% polyvinyl-pyrrolidone, and 1.0% sodium pyrophosphate. Labelled nuclear RNA was resuspended in a buffer containing 2x TESS plus 0.1 mg/ml tRNA, 0.02% Ficoll, 0.02% polyvinyl-pyrrolidone, and 0.1% sodium pyrophosphate. Samples to be compared were brought to the same concentration of radioactivity by adjusting solution volumes, and 1-2 ml of each solution was applied to a prehybridized replicate filter. A dilution of 1 to 1000 was used for measuring 28S rRNA in a separate hybridization. Filters were hybridized under paraffin oil at 65°C with rotary shaking for 35-40 hours. Filters were washed in 2x SSC at 65°C and treated for one hour at 37°C with pancreatic RNase (0.4 mcg/ml) and T1 RNase (10 U/ml) followed by proteinase K (50 mcg/ml in 150 mM NaCl, 1 mM EDTA, 1 mM Tris (pH 7.4), 1% SDS) for one hour at 37°C, with rinses (2xSSC) after each treatment.

AUTORADIOGRAPHY AND DENSITOMETRY

Pre-flashed (Laskey and Mills) Kodak XAR-5 film was

exposed to washed filters at -70°C with intensifying screens. After development, signal intensity was measured with a Helena Quick-Scan Jr. densitometer. In the course of numerous experiments, the relative transcription rate of CHO-B was essentially invariant in different preparations of normal hepatocyte nuclei from tissues or cultured cells (unpublished observations) and I 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 labelled RNAs, quantitative data from densitometric analysis in some experiments (Tables 9 and 12) was normalized to set the CHO-B signal equivalent in all samples.

CHAPTER THREE

CHANGES IN LIVER-SPECIFIC COMPARED TO COMMON GENE TRANSCRIPTION DURING PRIMARY CULTURE OF MOUSE HEPATOCYTES.

If mature liver cells are autonomously and irreversibly determined to transcribe liver-specific mRNAs at a high rate, exposing the cells to altered environments should have minimal effect on the differential transcription of liver-specific mRNAs compared to common mRNAS (i.e., those with a widespread tissue distribution). If liver cells require specific external signals or conditions for maintained high rates of liver-specific transcription, then perturbing the normal cellular environment might be expected to result in changes in liver-specific transcription. And, especially important, if mechanisms exist which coordinate the transcription of genes related only in their tissue-specific pattern of expression, under some circumstances changes in the transcription of one liver-specific mRNA should be paralleled by changes in many others.

As reviewed in the Introduction, many investigators had observed that hepatocytes suffer declines in various liver functions after several days in primary culture. However, the biochemical levels of control and molecular mechanisms responsible for these changes had never been examined. Nor had the resulting levels of liver function been compared in a

clear and quantitative manner to the levels of function seen in the normal liver. And nor had a sampling of liver-specific functions as broad as that represented by the plasmid collection produced by Derman, et al. (1981) been simultaneously monitored in primary cultures of hepatocytes.

To determine whether or not disaggregating the liver and exposing the cells to a standard tissue culture environment would result in significant, coordinated changes in liver-specific gene expression, I measured both the concentration and nuclear transcription rate of various liver-specific mRNAs as well as several common mRNAS in hepatocytes at several points during primary culture and in the intact liver.

ESTABLISHMENT OF CULTURES

Hepatocyte cultures were prepared by collagenase perfusion, as described in Materials and Methods. Single cells plated in a culture medium supplemented with insulin, hydrocortisone and 10% fetal calf serum attached to plastic culture dishes within 2 hours. After attachment, the cells flattened and spread, establishing apparent intercellular contacts within the first 24 hours. Although the cells continued to extend processes, the cell number was stable for at least one week, with no observed mitotic figures or any significant change in the number of nuclei per unit area in the culture dishes (assessed by light microscopy). At least 70% of the cells appeared to be binucleate. Other aspects of

the characteristic hepatocytic morphology (Klaunig, et al., 1977a, 1977b; Seglen, 1976) gradually gave way to an elongated morphology after several days. By 10 days in culture, it was difficult to judge by morphology whether the cells on the dish were in fact originally hepatocytes.

To assess the overall levels of protein and RNA synthesis, parallel cultures from a single mouse liver were labeled upon establishment of the culture or 1, 2, or 4 days later with ^{35}S-methionine or ^{3}H-uridine (Table 4). In addition, the total cell protein per culture dish was determined and remained essentially constant through the first 4 days. The incorporation of ^{35}S-methionine increased shortly after the cells were plated and was similar at 1, 2, and 4 days of culture. ^{3}H-uridine incorporation was lower during the first 24 hours and had increased at 2 and 4 days of culture. In other measurements (data not shown) a comparable level of ^{35}S-methionine incorporation was obtained in a 19-day-old culture.

Also, two-dimensional gel analysis of protein extracts from intact livers (labeled by intraperitoneal injection of ^{35}S-methionine) and from cells labeled during the first 2 hours, the third hour, the eight hour and the 24th hour in culture revealed changes in labeling intensity of some spots, but a general stability in the protein synthetic profile of most of the abundant proteins.

Table 4: Macromolecular Synthesis Rates and Total Protein in Hepatocyte Cultures.

Time	Total protein (mg)	^{3}H-uridine incorp.		^{35}S-met incorp.	
		Total	per mg	Total	per mg
2 h	15.0	43,600	2,910	837,000	55,800
1 d	13.3	32,190	2,420	4,059,600	305,000
2 d	12.0	105,350	8,750	4,252,500	354,000
4 d	12.9	69,700	5,400	4,559,100	353,000

16 replicate cultures were established from a single mouse liver. At the indicated times after plating, duplicate cultures were labeled for 30 min as described in the text. Total protein was determined for the uridine-labeled plates. Data presented are averages of data obtained from the duplicate cultures.

These results suggest that the cells maintain general macromolecular synthetic capacities. Most of the cells remain viable, and no replication is evident, at least within the first few days of culture.

mRNA CONCENTRATIONS

Total cellular RNA was extracted from fresh livers and from cells after various periods in culture, and the fraction of poly(A)$^+$ RNA was isolated by oligodeoxythymidylate chromatography. The presence of specfic mRNAs was determined in equal portions of the various poly(A)$^+$ RNA samples by the Northern blot procedure (Materials and Methods), involving electrophoresis through denaturing agarose gels, transfer to nitrocellulose and hybridization to ^{32}P-labeled cDNA probes. cDNA probes used in the assay included the 11 liver-specific clones isolated by Derman et al. (1981), probes that hybridized to the mRNAs encoding albumin or alpha-fetoprotein, and DNA samples containing sequences complementary to five mRNAs that are common to a variety of tissues.

By the Northern blot assay, the initial liver cell suspensions before plating contained amounts of all liver-specific mRNAs approximately equal to that found in whole livers (Fig. 2). Moreover, the levels of all the liver-specific mRNAs remained high through day 1 or 2 of culture. Small variations between 6 and 24 hours in several mRNAs, e.g., plivS-6, were not further investigated although they

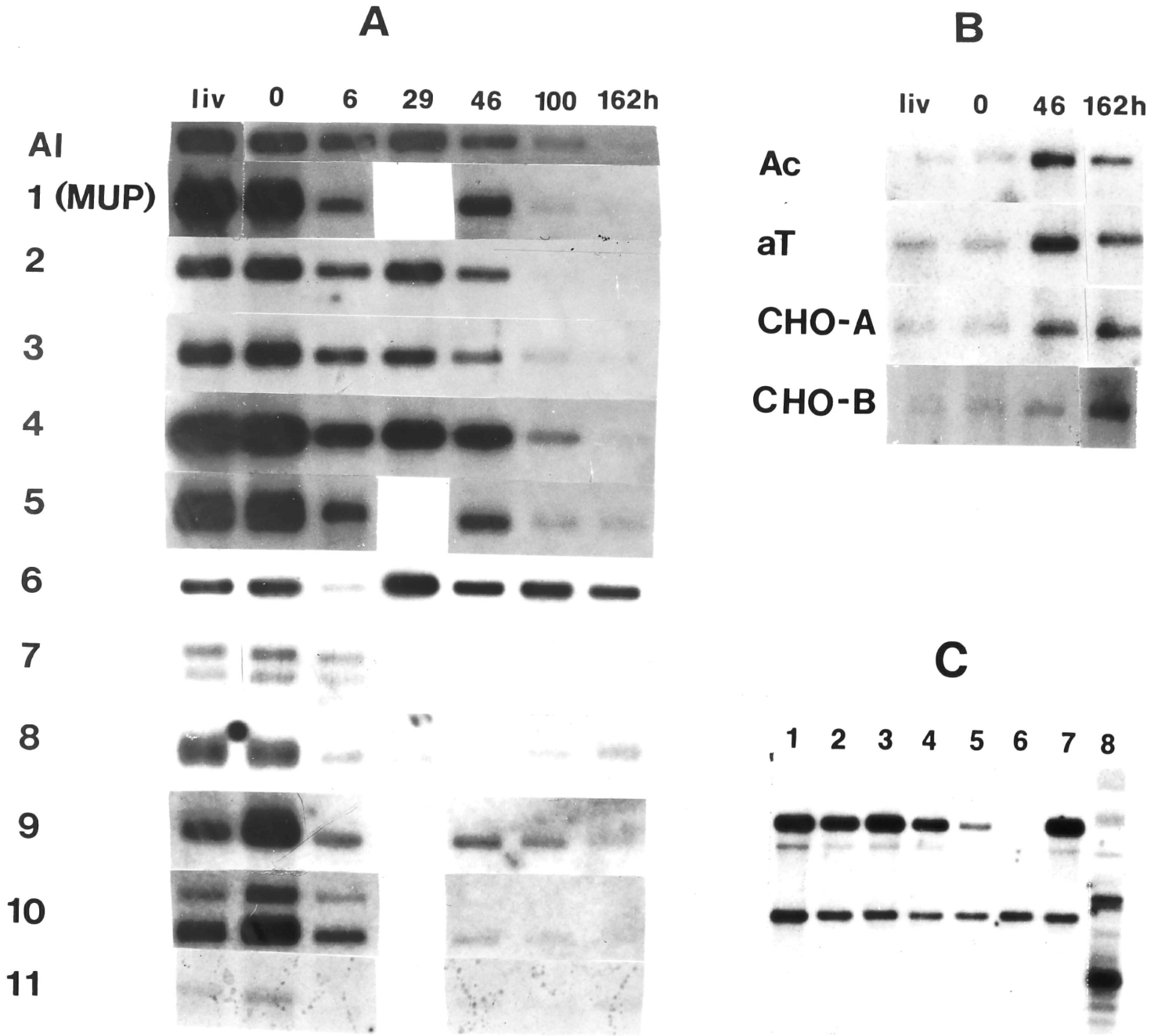
Figure 2. Northern blot analysis of specific RNA concentrations in hepatocytes cultured through 1 week.

Cultures from 2 mice were established, and RNA from 2 plates was isolated at the indicated hours after plating. Whole liver RNA is designated liv, and freshly isolated hepatocytes are 0. Equal amounts of poly(A)+ RNA (measured by optical density), ranging from 0.1 to 1 mcg according to the abundance of the mRNA to be measured, were loaded into each well of a denaturing agarose gel, and after electrophoresis the RNA was transferred to nitrocellulose and hybridized to a single nick-translated cDNA probe (two probes in [C]). pBR322 DNA digested by HincII, EcoRI and HinfI was included on each gel to provide size markers, as shown in [C]. Each series of bands for a specific mRNA was cut from autoradiographs and compiled for (A) and (B).

(A) Liver-specific RNAs: probes abbreviated as in Table 1. Blanks represent gel slots where no RNA sample was loaded.

(B) Common RNAs: probes abbreviated as in Table 2.

(C) photograph of an entire representative blot, hybridized to the albumin probe (upper band) and to a cDNA clone which hybridizes to two mitochondrial RNAs (lower bands). This figure indicates the change in albumin concentration despite the constancy of mitochondrial RNA in the input. Lanes 1 to 6, from 0h to 162 h (as in A); lane 7, whole liver RNA; lane 8, pBR322 size markers.


A
liv 0 6 29 46 100 162h
AI
1 (MUP)
2
3
4
5
6
7
8
9
10
11
B
liv 0 46 162h
Ac
aT
CHO-A
CHO-B
C
1 2 3 4 5 6 7 8

were observed in other experiments as well. Since the mRNA concentration of the cells after a day or two in culture closely resembled that found in whole livers or in cells just after removal from the animal, the process of perfusion, disaggregation and initial cell culture appeared to result in a population of cells that functionally resembled the population of hepatocytes in the normal liver.

Continued culture of the hepatocytes, however, showed that, with the single exception of plivS-6, the levels of all liver-specific mRNAs declined detectably within 48 hours. Most liver-specific mRNAs continued to decline over the next several days and became virtually undetectable by day 7. In contrast, at the end of 1 week in culture, the cells contained greater levels of all the common mRNAs and virtually identical levels of plivS-6 compared with freshly dissociated cells or the cells of whole livers. The time course of the described changes varied somewhat with different mRNAs. Some liver-specific mRNAs (plivS-7, -8, and -11) declined quickly and remained at constant but reduced levels, whereas others (albumin, plivS-1, -2 through -5, and plivS-10) showed a steady continuous decline beginning sometime after day 1. The signal for alpha-fetoprotein (not shown) was very low even in whole livers and freshly isolated cells, which is not surprising considering that the mature hepatocyte is known to make only small amounts of this protein. Nevertheless, mRNA concentrations for alpha-fetoprotein also appeared to diminish during culture.

The time-course of these changes in liver-specific mRNA concentrations is similar to the time course commonly observed for loss of liver function in cultured hepatocytes (e.g., Michalopoulous and Pitot, 1975, Pitot and Sirica, 1980; Newman and Guzelian, 1982).

TRANSCRIPTION RATES

To determine whether these changes in mRNA concentration were controlled or paralleled by changes in specific gene transcription, transcription analyses of nuclei from cultured hepatocytes and fresh liver were performed using the various liver-specific and common cDNA probes. The transcription assay based on nascent labelling of isolated nuclei, dot hybridization and autoradiographic detection was performed as described in Material and Methods.

Three sets of autoradiographs are presented. In the first experiment (Fig. 3A), the transcription of most liver-specific genes had declined significantly after cells had been in culture only 24 hours. In the same experiment (not shown), cells after 10 days in culture had a very similar transcriptional profile to the cells assayed at 24 hours in culture.

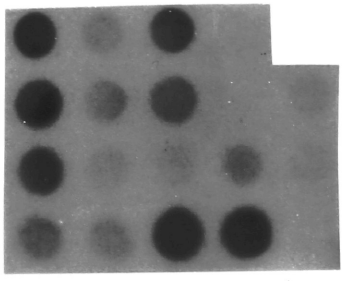
In the second experiment (Fig. 3B), the rate of decline of liver-specific transcription was defined more precisely by examining cells after 7, 14 or 24 hours in culture. The signal for plivS-1 (major urinary protein or MUP) declined to background levels within the first 7 hours of culture, but

Figure 3. Dot hybrid analysis of specific gene transcription in cultured hepatocytes.

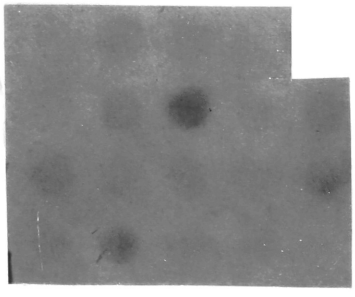
Nuclei of liver cells were prepared with detergent after perfusion of the liver with cold medium at 4 °C (A). The nuclei of plated cells were prepared in the same manner at the times described in the text. The sample marked liver in (B) was prepared by sedimentation of nuclei through sucrose without detergent (Materials and Methods). Nuclei were labelled and hybridized to plasmid DNAs as described in Materials and Methods. The key at the bottom indicates the arrangement of plasmid DNAs on the filter (see Tables 1 and 2).

A

cold perfused liver



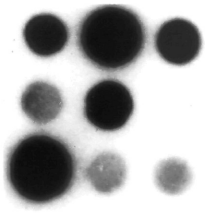
24 h



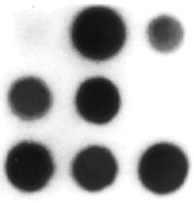
1	5	9		
2	6	10	pBR	aT
3	7	11	aFP	bT
4	8	Al	Al	

B

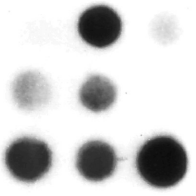
liver



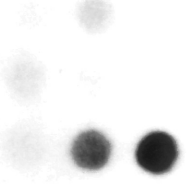
7h



14 h



24 h



1	2	4
9	11	pBR
Al	aT	Ac

transcription of the other liver-specific genes followed a more gradual pattern of decline over the 24 hour period. On the other hand, transcription of actin and tubulin mRNA sequences increased substantially during the first few hours of culture and remained higher at 24 hours than in the intact liver.

Several additional points can be made from the third experiment (Fig 4). First, transcription of MUP was already reduced after only 2 hours of culture, whereas transcription of actin sequences increased dramatically at two hours (a quantitative change of 50-fold or more by densitometric analysis) before returning to and remaining at normal levels between 24 and 96 hours. The significance of this large transient change in actin transcription is not clear, but it may be related to the changes in attachment and cell shape that the cells undergo during dispersion and cell culture (Farmer et al., 1983). Transcription of other common mRNA sequences remained relatively constant throughout the four day culture period.

Three liver-specific sequences (plivS-6 and -7 in Fig.4, and plivS-8 in the experiment shown in Fig. 3A) showed little decrease at any point through at least 4 days in culture. The stability of plivS-6 transcription is consistent with the results of the Northern blot analysis of mRNA concentrations, but both plivS-7 and -8 showed substantial declines in concentration in cells after a day in culture (Fig. 2). A similar lack of correlation between transcription rate and

Figure 4. Dot hybrid analysis of specific gene transcription rates in hepatocytes.

Nascent-labelled nuclear RNA from whole liver (liv), freshly isolated hepatocytes (Ø) and cultured hepatocytes (at the indicated hours after plating) was prepared and hybridized to plasmid DNAs as described in Materials and Methods. The columns marked + indicate the addition of 1 mcg of alpha-amanatin per ml during the RNA labeling (liver and 24 h samples only). (A) RNA polymerase II products. (B) RNA polymerase I and III products.

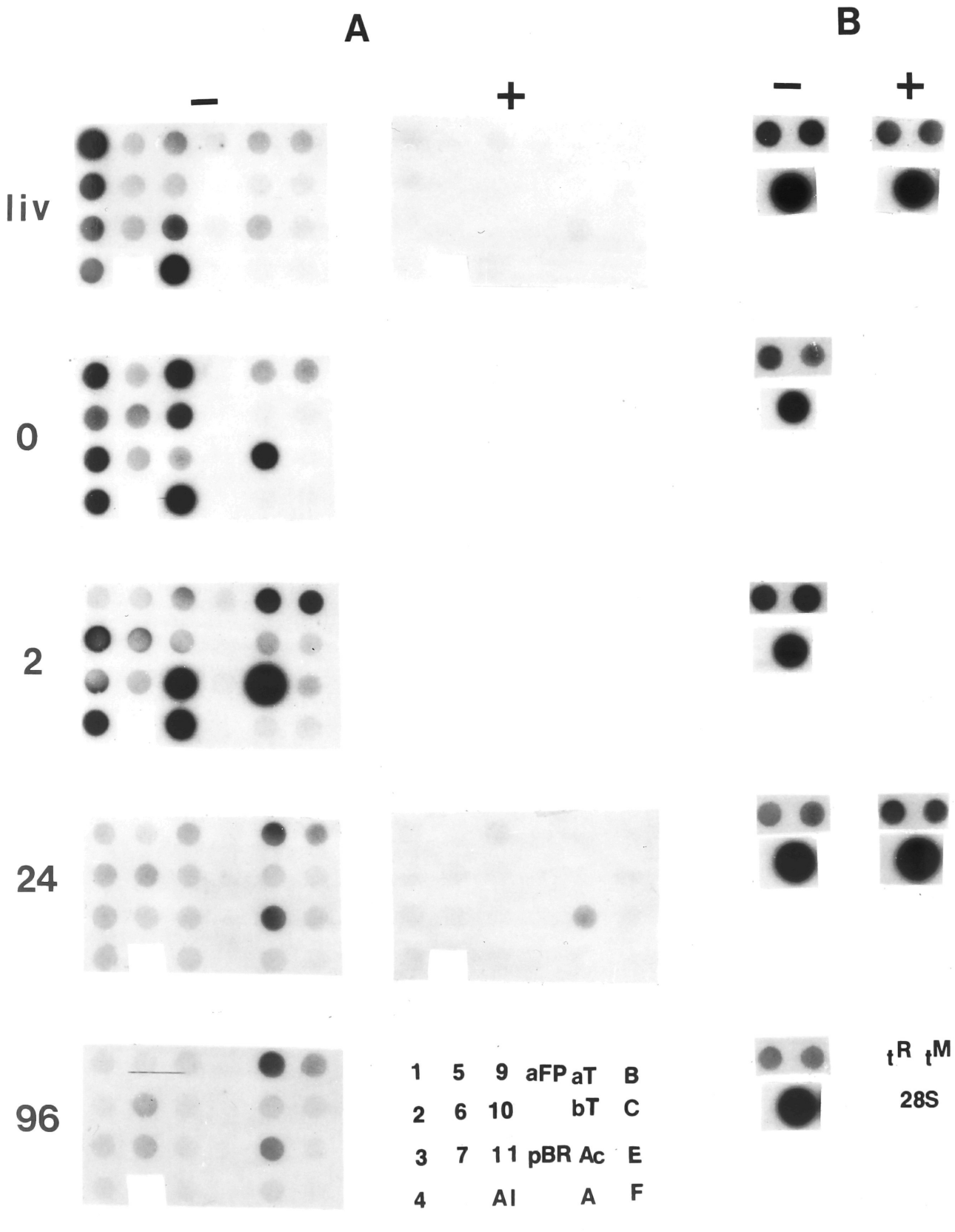

A
B
−
+
−
+
liv
0
2
24
96
1 5 9 aFP aT B
2 6 10 bT C
3 7 11 pBR Ac E
4 Al A F
tR tM
28S

Table 5: Changes Relative to Intact Liver in Specific Gene Transcription Rates.

	2h Fig. 4	7h Fig. 3B	14h Fig. 3B	24h A	24h B	24h C	24h Avg	96h Fig. 4
AL	0	-3	-7	-25	-30	)-50	-50	)-50
1	-20	-25	-50	-20	-50	)-50	-50	-50
2		-2	-6	-10	-50	-50	-37	-30
3				-6	-20		-13	-8
4		-3	-17	-4	-20	-30	-18	-7
5				-2	-4		-3	-3
6				+0.5	-4		-1.5	+0.5
7				-2.5	-6		-4	0
8					-2		-2	
9		+1.5	-2	-2	-30	-10	-14	-4
10				0	-2		-1.5	-3
11	+5	0	-5	-10	-6	-30	-15	-25
Ac	)+50	+7	+6			+2	+2	+2
aT	+6	+3	+2	+2	0	+1.5	+1.5	+2
bT	+5			0			0	0
A				+4			+4	+4
B	+4			0			0	0
C, E, F				0			0	0

Autoradiographs from Figs. 3 and 4 were analyzed by densitometry. Data expressed as factor change in signal for each probe (left column) relative to signal in intact liver. 24h: A - Fig. 4; B -Fig. 3A; C - Fig. 3B

concentration for these two mRNAs was later observed in an analysis of liver-specific mRNA production in the fetal liver (Powell, et al., 1984).

One other transient variation was also noted. The transcription signals for plivS-9, -10 and -11 increased in nuclei from cells just after disaggregation, compared with transcription in nuclei obtained from whole livers. These changes were apparently reflected in transiently increased mRNA concentrations (Fig. 2). By 24 hours in culture, however, the transcription of these genes had declined. In numerous additional experiments (e.g., next Chapter), transient increases in transcription of these sequences have sometimes but not always been observed in freshly isolated hepatocytes.

By comparing densitometric tracings of the autoradiographic exposures (Table 5) and by comparing different exposures and RNA inputs (Materials and Methods), the decrease in signal intensity between 0 and 96 hours was estimated to be 10-fold or more for MUP, albumin, plivS-2, -3, -4 and -11, and about three-fold for plivS-5, -9 and -10. (More refined quantitation of the change in transcription of specific genes is presented in Chapter 5).

EFFECT OF ALPHA-AMANATIN ON TRANSCRIPTION RATES.

To establish that the labeled nuclear RNA that hybridized to the cloned probes was the product of RNA

polymerase II transcription, nuclei isolated from whole livers and cultured cells were divided and labeled with or without 1 mcg of alpha-amanatin per ml, a dose which specifically inhibits RNA polymerase II (Roeder, 1976). As shown in Fig. 4, a-amanatin effectively eliminated the production of hybridizable RNA from both the liver-specfic and common genes. A low level of residual transcription of certain genes that are presumably transcribed by RNA polymerase II (in particular, the actin sequences) is evident and accords with the ~90% reduction expected (Roeder, 1976).

Because transcription rates were compared by hybridizing an equal amount of the labeled RNA, the relative levels of hybridization might conceivably be misinterpreted if the proportion of activity of the three polymerases varied significantly. To test this, labeled nuclear RNA (Fig. 4B) was also hybridized to cloned DNA samples that encode 28S rRNA or tRNA(arg) and tRNA(met) sequences. The hybridization to these probes was essentially constant, with equal input of radioactivity from different samples indicating a constant input of polymerase II product in the various samples. In addition, the transcription of 28S and tRNA sequences was unaffected by a-amanatin at 1 mcg/ml.

CONCLUSIONS

These results establish that quantitatively large decreases in the production of many different liver-specific occur in hepatocytes when they are dispersed from the normal

intact tissue and cultured as a monolayer under standard culture conditions. Importantly, the production of several common mRNAs (and 1 of 13 liver-specific mRNAs, encoding transferrin) does not decrease. Because changes in transcription preceded changes in mRNA concentrations, and were of a similar magnitude, the loss of liver-specific mRNAs apparently resulted from a decline in their transcription rates. No evidence for significant levels of cell death, excessive stress or selection was obtained from morphological observation, metabolic labeling, two-dimensional protein analysis, and Northern blot analysis of mRNA concentrations. Rather, a specific change seems to be occurring within the isolated hepatocytes themselves.

Although some variations were observed in the exact time course of these changes for different liver-specific genes, the general pattern of change was very similar for most of the liver-specific genes examined. This strongly suggests that some mechanism of coordinate control is involved in the regulation of many (and probably most) liver-specific genes. Furthermore, this mechanism must somehow be sensitive to the process of tissue disaggregation and primary cell culture. In the next two chapters, the exact nature of this relationship between changes in tissue-specific transcription and primary cell culture is examined further.

One final caveat should be noted. With some of the cloned cDNA probes used in these assays, their genomic counterparts have been identified, and in some cases multiple

cross-reactive transcription units have been shown to exist (e.g., MUP, Shahan and Derman, 1984). One cannot be certain, therefore, that the output of the same transcription unit is always being measured in different circumstances. Similarly, the significance of the residual low level of liver-specific transcription measured in the cultured hepatocytes after a day or so in culture is uncertain. In fact, similar low-level "liver-specific" transcriptional signals have been obtained in some cases with nuclei from mouse erythroleukemic cells (data not shown) and with nuclei from other tissues (Powell, et al., 1984). This last point will be addressed again in Chapters 6 and 7.

Nonetheless, the central conclusion is clear: common gene transcription continues, but trancription of most liver-specific genes declines when liver cells are isolated from the tissue and placed in culture. The total transcriptional apparatus has not been dismantled, but a differential effect has resulted.

CHAPTER FOUR

EFFECT OF CHANGING CELL CULTURE CONDITIONS ON LIVER-SPECIFIC mRNA PRODUCTION IN ISOLATED HEPATOCYTES

Why does liver-specific transcription decline so dramatically in isolated cultured hepatocytes? There are three obvious and general explanations. One is that hepatocytes might require specific humoral conditions or factors to maintain high rates of liver-specific transcription, and these conditions are not adequately reproduced by the artificial culture medium. Perhaps a specific hormone is absent, or the balance of nutrients is not right, or there may be inhibitors of differentiated function in fetal calf serum, for example.

A second possible explanation is that the cells require a specific signal for maximum tissue-specific function which they normally receive through cell-cell contacts or through contact with the extracellular matrix. If this were the case, culturing the cells with other non-parenchymal cell types, or at a high density, or on an extracellular matrix might result in maintained liver-specific transcription in vitro.

The third explanation is more complex: perhaps hepatocytes do not require a discrete signal for continued differentiated function at all, but must be maintained in a critical geometry or organizational state for maximum tissue-

specific transcription to proceed. This critical organizational state might depend on the geometry of extracellular contacts, for example, and it might arise or evolve slowly as the tissue and the hepatocytes mature. If maximum transcription is linked directly to the organization of cells in a tissue, it could be very difficult to define conditions for maintaining maximum differentiated function *in vitro*.

Each of these three explanations implies somewhat different cellular mechanisms for the coordinated control of tissue-specific transcription, as well as for the control of cell differentiation during development. In an attempt to better understand the precise cause of the observed loss of liver-specific transcription, I conducted a series of experiments seeking to define culture conditions that would lead to an improvement in liver-specific transcription in isolated hepatocytes.

EXPERIMENTAL DESIGN

In each experiment, transcription rate profiles (obtained using the dot hybrid assay described earlier) were compared for nuclei isolated from fresh liver, from cells cultured under the standard conditions described in the last chapter (Dulbecco's modified essential medium plus insulin, hydrocortisone and 10% fetal calf serum), and from cells under one or more sets of test conditions. Since the

decline in liver-specific transcription occurred mostly in the first 24 hours of primary culture under standard conditions, in most experiments cultures were assayed after 24 hours. Where there was reason to suspect that a longer exposure to the test culture condition might be necessary for improved cellular function (e.g., attachment to extracellular matrix, selection in arginine-free medium) cells were also assayed after as much as a week in culture. As much as possible, cultures to be compared in any one experiment were established and maintained in parallel (often from the same mouse liver cell preparation). A fresh preparation of nuclei from intact mouse livers (or in some cases from cells immediately after collagenase dispersion) was always used as the positive control in the transcription assay.

Each dot hybrid assay of transcription included both liver-specific cDNA probes and common probes as controls, with hybridization to pBR322 vector DNA used as a measure of non-specific background hybridization. In most experiments, analysis of net polymerase II transcription (i.e., by measuring polymerase I activity with a 28S rRNA probe and polymerase III with a tRNA probe) was also conducted.

CELL CULTURE CONDITIONS EXAMINED

A wide range of culture conditions was examined, as summarized in Table 6. Manipulations included alteration of the basal medium, the amount and type of serum and hormones added, the substrate to which the cells were attached,

exposure to oxygen, the addition of physiological and non-physiological inducers and drugs, the inclusion of other cell types in the cultures, and cell density. The rationale for each manipulation is summarized briefly in the following paragraph.

The medium of Enat, et al. (1984) was developed to improve function in rat hepatocytes, and contains a mixture of hormones, factors and no serum (see Materials and Methods). Testosterone, EGF (epidermal growth factor) and glucagon have numerous effects on liver cells (e.g., Guguen-Guillouzo and Guillouzo, 1983; Miller and Griffin, 1975) and are in low concentration in fetal calf serum. Hydrocortisone has a short lifetime in culture (Miller and Griffin, 1975) and has been considered critical in maintaining hepatocytic function (Greengard, 1975; Guguen-Guillouzo and Guillouzo, 1983). Exchange of soluble metabolites or gases was manipulated by changing feeding frequency or medium depth (over 40 ml of medium in a dish was lethal). DMSO (Higgins and O'Donnell, 1982), retinoic acid (Strickland, 1979), (dibutyryl) cyclic AMP (e.g., Ruiz-Bravo and Ernest, 1982), butyrate (Kruh, 1982), cycloheximide (Nevins, 1981), calcium (Hennings, et al., 1980) and early products of adenovirus (Nevins, 1982) have been used variously as inducers in hepatocytes, hepatomas or similar epithelial cell systems. A23187 is a calcium ionophore with inducing properties in some cells (Bridges, 1981). Demecolcine, a colchicine analog, (Crossin and Carney, 1981) and cytochalasin B (Weber,

1976) affect cytoskeletal organization and function, and vanadate is a generalized inhibitor of phosphorylases which affects intermediate filament organization (Wang and Choppin, 1981) among other things. Concanavalin is a lectin that may cross-link cell surface components, decreasing their mobility and influencing intracellular organization (Gall and Edelman, 1981). Fetal cells have lower liver-specific transcription rates than adult cells (Powell et al., 1981), but these rates declined in culture like adult monolayers, whether in basal or arginine-free (Leffert et al., 1977) medium. Endothelial, epithelial, and other non-parenchymal cells are constituents of liver tissue that may be lost when hepatocytes are isolated, and may have inducing properties in co-culture systems (Guguen-Guillouzo et al, 1983; Bissell, 1983). Collagenous substrates are generally considered to improve cell function in vitro (Kleinman, et al., 1981; Wicha, et al., 1982), and biomatrix is a complex collagenous substrate developed for hepatocytes (Reid, et al., 1984; Enat, et al., 1984). Cell density was varied between 2-16 x 10^4 cells/cm^2 (Nakamura, et al., 1983).

EFFECT ON TRANSCRIPTION

Isolated hepatocytes exposed to any of the culture conditions described above and in Table 6 had similar, low transcription profiles for the panel of tissue-specific mRNAs. In all cases, the transcriptional profile was essentially indistinguishable from that presented in the

previous or the following chapters for isolated hepatocytes cultured in monolayers under basal conditions. In some experiments, the transcription rate of one or two liver-specific genes varied by factors of 2-4 (up or down) in the test cultures compared to the control cultures, but these variations were usually irreproducible, and in no case was a general effect seen on the liver-specific transcription units as a group. The most substantial variation in transcription was observed in comparisons of cells plated at different densities: transcription of plivS-2 and Albumin was about 4-fold lower at the lowest compared to the highest cell density, but other genes showed no effect, and all liver-specific signals remained far below signals for intact liver at all culture densities.

Table 6: Culture Conditions with Equivalent Effects on Liver-Specific Gene Transcription in Mouse Hepatoctyes.

Conditions	Medium	Hormones	Other Additives	Substrate	Time
Basal	Dulbecco's	Insulin Hydrocor-tisone	10% Fetal calf ser.	tissue culture plastic	1-10 d
Horse Serum	"	"	10% horse s.	"	24 hr
20% Serum	"	"	fetal calf s.	"	"
50% Serum	"	"	"	"	"
Hormonally Defined	RPMI 1640	Enat, et al.	Enat, et al.	"	1-6 d
No Hormones	basal	none	basal	"	24 h
Testoster-one, EGF, Glucagon, Retinoic Acid	"	basal + (Exp. Proc.)	"	"	"
Hydrocort-isone re-placement	"	basal+ HC every 5 hours	"	"	"
change medium 5x	"	basal	"	"	"
medium depth: 10-40 ml	"	"	"	"	"
95% O2 5% CO2	"	"	"	"	"
DMSO	"	"	basal+	"	1-4 d
Retinoic Acid	"	"	basal+	"	24 h
cyclic AMP	"	"	basal+	"	1-4 d
Ret. Acid + cAMP	"	"	basal +	"	3 d

Table 6, cont.

Conditions	Medium	Hormones	Other Additives	Substrate	Time
5 mM Na butyrate	basal	basal	basal+	basal	24 h
Cyclohex-imide	"	"	basal+	"	"
3x CaCl2	"	"	basal +	"	"
A23187	"	"	basal+	"	"
no EDTA,	"	"	basal	"	"
Ad5 infec-tion, 6-12 h	"	"	basal + Ad5 @ 36h	"	42-48 h
Vanadate	"	"	basal+	"	24 h
Cytochal-asin B	"	"	basal +	"	"
Demecoline	"	"	basal+	"	"
Concanav-alin A	"	"	basal +	"	"
suspension culture	"	"	0-50% fetal bov., calf or horse ser.		1-2 d
18 Day Fetal Cells	"	"	basal	"	1-5 d
Fetal Cells Arg- medium	Exp. Proc.	"	dialyzed 10 % fcs	"	"
+ bovine aortic endo-thelial c.	"	"	basal + equal # BAEC	"	"
+ rat liver epithelial cells	Williams D	"	basal + equal vol. LEC	"	"
Williams D medium	"	"	basal	"	"
+ mouse liv. non-paren-chymal cells	"	"	"	"	24 h
plastic substrate	"	"	"	Nunc or Falcon	24 h

Table 6, cont.

Conditions	Medium	Hormones	Other Additives	Substrate	Time
rat liver biomatrix substrate	basal "	basal "	basal "	biomatrix on petri dishes	24 h
universal biomatrix	"	"	"	"	3 d
universal biomatrix, def. med.	RPMI 1640	Enat et al.	Enat et al.	"	6 d
rat liver biomatrix, def. medium	"	"	"	"	7 d
liver extract	basal	basal	basal+	"	24 h
cell density	"	"	"	"	"

Hepatocytes were prepared, cultured and assayed for tissue-specific transcription as described in Materials and Methods, where additional details of experimental manipulations are given. In each experiment, cultures were compared to normal intact liver and to cells cultured for the same time under basal conditions. Liver-specific transcription patterns in cells cultured under all of these conditions were essentially indistinguishable, although occasional small (2-3 fold) variations in transcription of particular genes in some experiments were observed. Where additional ingredients have been added to the culture medium, a "basal+" is entered in the chart. Dosages were determined based on the literature and on observation of cell morphology.

POST-TRANSCRIPTIONAL EFFECTS

Although none of the culture conditions significantly improved tissue-specific transcription in isolated hepatocytes, some of them may have had other post-transcriptional effects on the cells. In most cases this possibility was not examined directly, but in one case there was enough evidence of a significant post-transcriptional effect to warrant further study.

The hormonally-defined medium of Enat, et al. (1984) had been developed in the laboratory of L. Reid at Albert Einstein College of Medicine. With her colleague, D. Jefferson, she had found by Northern blot analysis evidence of a substantial increase in albumin mRNA concentration in rat hepatocytes cultured in this medium (called "HDM" for hormonally-defined medium").

We therefore jointly conducted an analysis of both transcription rate and mRNA concentration in rat hepatocytes cultured in HDM or serum-supplemented medium (SSM), and compared to the intact rat liver (note: SSM does not contain the insulin and hydrocortisone added to the "basal" culture medium described earlier). The transcription analysis included probes for most of the liver-specific and common mRNAs described so far. Probes used in the Northern blot analysis included albumin, plivS-2, -3, -4 and -5, and actin and tubulin plasmids. Cells were cultured for the first 24 hours in a mixture of SSM and HDM, and for the remainder of

the period in one or the other medium. The results are described in detail in Jefferson, et al. (1984), and are summarized below and in Table 7:

Albumin mRNA concentration in cells cultured for 5 days in HDM was equivalent to its concentration measured in fresh intact liver, after a small decrease measured at the second day. However, the transcription rate of albumin mRNA was measured to be about 8 to 9-fold lower in these cells than in the intact liver. In SSM, however, albumin mRNA concentration fell more than 200-fold in 5 days and its transcription rate fell about 20-30 fold. Therefore, culture in HDM significantly increased albumin mRNA concentration compared to culture in SSM, but primarily through post-transcriptional mechanisms which resulted in an apparent albumin mRNA stability (or processing efficiency) that was higher even than in the normal intact liver.

Post-transcriptional enhancement of plivS-3 (alpha-1-anti-trypsin) mRNA was also observed, although it was quantitatively less profound. No post-transcriptional effect was observed for the other liver-specific genes examined, however, implying that this effect may not involve coordinate regulation of liver-specific genes, but individual regulation of a few isolated genes. Also, no increase in transcription rate in HDM compared to SSM was observed for any of the mRNAs examined except albumin.

Evidence of possible post-transcriptional modulation of

common mRNAs in the cultured hepatocytes was also obtained in the same set of experiments. mRNAs encoding actin and tubulin increased in concentration several hundred to a thousand-fold in the first two days of culture in either medium, and dropped only by a factor of about 2 after three more days of culture. Yet the transcription rate of these mRNAs was little or no higher in these cells after 2-5 days in culture than in the normal liver. Earlier culture times were not examined in this experiment, and this change could result solely through transcriptional means if the half-life of these mRNAs were normally about 2 to 3 days, and if their total transcriptional output increased about 1000-2000-fold in the first day of culture and then declined to normal. In fact, based on results presented in the previous chapter (and Clayton and Darnell, 1983) a large but very brief increase in actin (and to a lesser degree tubulin) mRNA transcription would have been expected in the first few hours in culture. In mouse hepatocytes (Fig. 4) this increase did not appear to be much more than a hundred-fold, although the quantitative accuracy of the dot-hybrid transcription assay over ranges greater than a hundred-fold has not been well defined (see Materials and Methods). Thus significant increases in the stability or processing of actin and tubulin mRNAs appear to result following cell culture in either medium, although rigorous demonstration of this conclusion will require further analysis.

Table 7: Effect of Different Culture Conditions on mRNA Concentrations and Transcription Rates in Rat Hepatocytes

2 Days in Culture				
mRNA	Relative mRNA concentration		Relative transcription	
	SSM	HDM	SSM	HDM
Albumin	-3.9	-3.1	-19.1	-9
plivS-3	-3.7	-1.7	-3.3	-4.3
Actin	+1000	+450	+3	+1.4
Tubulin	+350	+220	1	1

5 Days in Culture				
mRNA	Relative mRNA concentration		Relative transcription	
	SSM	HDM	SSM	HDM
Albumin	-250	1	-32	-8
plivS-3	-30	-6	-2.6	-3.3
Actin	+690	+220	1	1
Tubulin	+120	+78	-2	-4

Data presented as change relative to rat liver, e.g., -3.9 is 3.9 times less than liver. From densitometric analysis of autoradiographic data. Multiple exposures were used to compare very high and very low signals. Transcription data includes small corrections for non-specific hybridization to pBR322 and normalization to constant 28S rRNA signal.

WHOLE ANIMAL EXPERIMENTS

Two experiments were also conducted with whole animals to address the following questions: 1) could biliary secretion into the cell culture medium be the cause of the loss in liver-specific transcription in isolated hepatocytes? 2) could a transient decrease in protein synthesis rates result in lost liver-specific transcription?

Biliary Secretion: hepatocytes normally secrete bile and various metabolites into the biliary tract, where they are eventually carried to the digestive tract for elimination from the body. However, cultured hepatocytes obviously remain exposed to their own secretions. Although changing the culture medium frequently did not improve liver-specific transcription (Table 6), I decided to conduct the following experiment, with the help of J. Friedman, M.D. The bile duct was ligated in several mice, leading to a back-up of bile into the liver. After 24-48 hours, the transcriptional profiles of the livers from these mice were measured with the dot hybrid assay. Fluctuations in the transcription of various genes were noted, although the only change that occurred in all experiments was a significant (10-fold or more) decrease in the transcription of plivS-1 (MUP). Transcription of other liver-specific and control genes did not change in a consistent pattern. I concluded that, with the possible of exception of MUP, there was no evidence that the observed declines in liver-specific transcription were

caused by damage or repression due to exposure to biliary secretions.

Transient Decline in Protein Synthesis: I had found earlier (Table 4) that protein synthesis rates increased about 6-fold in the first two hours of primary culture. Whether or not this reflected a real increase in protein synthesis over the rate of the normal liver, or whether it reflected recovery to normal levels following a cell-disaggregation induced decrease (Ben-Ze'ev et al., 1980), was impossible to determine. If (hypothetical) factors which activate liver-specific transcription had a brief half-life and were auto-catalytic for their own synthesis, a transient decrease in protein synthesis could result in permanent loss of these factors in the cell, and a parallel loss in tissue-specific transcription. To investigate this possibility, I conducted the following experiment: mice were given intra-peritoneal injections of cycloheximide or emetine, at doses which were empirically determined to result in at least a six-fold decrease in ^{35}S-methionine incorporation into TCA-precipitable material in livers in vivo within two hours. 3-36 hours later the livers of the mice were assayed for liver-specific transcription as usual, and compared to livers from mice that had received control injections of saline. Except for plivS-1 (MUP), no significant and reproducible changes were observed in the transcription of the liver-specific mRNAs assayed. With cycloheximide injections, substantial and sustained decreases (at least 10-fold) in MUP transcription

were measured in livers 3, 15 and 36 hours after treatment. However, in the experiment with emetine injection, little or no decrease in MUP transcription was measured (at 3 or 15 hours post-injection). Based on these results, I concluded that there was no evidence that a transient loss in protein synthesis rate was the cause of the coordinate loss of liver-specific transcription in the isolated hepatocyte.

In the course of these experiments with protein synthesis inhibitors in whole animals, I noticed a substantial increase (perhaps two-fold) in the volume of the liver 36 hours after injection with cycloheximide. This increase may have been due to cell growth. If so, it might imply the existence of a short-lived suppressor of hepatocyte proliferation in vivo. I did not pursue these observations further.

In general, the experiments with whole animals were awkward, and difficult to interpret because of the many uncontrolled variables.

CONCLUSIONS

No culture conditions were found which prevented or reversed the decline in liver-specific transcription in hepatocytes isolated from the tissue. No evidence was obtained for a specific signal for liver-specific transcription, either communicated through specific humoral factors or through specific cell-cell or cell-matrix

contacts. However, such a negative result obviously does not disprove the existence of such signals. Also, the possibility remains that some of the culture techniques examined may exert some post-transcriptional effect on some liver-specific mRNAs. Such a phenomenon was observed for two genes (albumin and a-1-antitrypsin) when rat hepatocytes were cultured in a serum-free hormonally-defined medium (Enat, et al., 1984). However, other liver-specific genes were not affected, implying that this phenomenon is not central to the coordinate regulation of tissue specificity. Whole animal studies were used to show that increased exposure to biliary secretions or a transient decline in protein synthesis does not cause a coordinate loss of liver-specific transcription in intact livers.

Based on these results, the possibility was seriously considered that hepatocytes must be maintained in a normal tissue organization for continued maximum levels of liver-specific transcription.

CHAPTER FIVE

DEPENDENCE OF LIVER-SPECIFIC TRANSCRIPTION ON TISSUE ORGANIZATION

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*. With this in mind and the assistance of A. Harrelson, I explored the use of conventional tissue-slicing techniques (McIlwain, 1960; Bak, et al., 1980) as a way to prepare liver tissue for culturing.

PREPARATION AND CULTURE OF LIVER SLICES

Livers to be sliced were first perfused with tissue culture medium to wash out blood and to insure quick exposure of all the cells in the tissue to the culture medium. With a McIlwain Tissue Chopper, livers were then cut into columns 0.325 mm x 0.325 mm x 2-3 mm. The columns were therefore approximately 10 to 15 cells in diameter, or about half the size of the classic liver lobule. In most experiments the slices were placed in Erlenmayer flasks that were flushed with 95% O_2/5% CO_2 and gently shaken. For comparison, monolayers were also cultured under 95% O_2/5% CO_2.

Measurements of macromolecular synthetic rates (Table 8)

Table 8. Macromolecular Synthesis in Liver Slice Cultures.

	RNA				PROTEIN			
	cpm	mg	cpm/mg	%	cpm	mg	cpm/mg	%
2H	1452	1.8	807	100	113300	2.0	56650	100
26H	1713	1.6	1070	133	63800	1.4	45570	80
50H	1030	1.7	635	79	68220	1.9	35900	63

Mouse liver slices were divided into 6 equal aliquots for culturing. At the times indicated, medium in individual cultures was replaced for 30 minutes with medium containing 10 mcCi/ml of either ^{35}S-methionine or ^{3}H-uridine, as described in Materials and Methods. The slices were then washed and quick frozen in PBS. After thawing, they were polytron homogenized and assayed for incorporated label and protein content. In contrast to slice cultures, monolayer cultures were found to increase protein synthesis by six-fold and RNA synthesis by three-fold between 2 and 48 hours in culture (Table 4).

indicated that, unlike monolayer cultures (Table 4), slice cultures maintain low total rates of RNA and protein synthesis which decline after 24 hrs. Furthermore, the slices gradually become fragmented in shaking cultures, so that after 24 hours 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

However, since slice cultures could be maintained for 24 hours with moderately active total protein and RNA synthesis, which is the amount of time required to observe the decline in liver-specific transcription in monolayer cultures, slice and monolayer cultures after 24 hours and fresh intact mouse livers were compared using the dot hybrid transcription assay. Autoradiographs from two such experiments are shown in Figures 5 and 6, and quantitation of the autoradiographic signals is given in Tables 9 and 10.

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 nuclei from slices usually averaged from 20 to 50% of that in liver nuclei for most genes, and was approximately five- to twenty-fold higher than in dispersed

Figure 5. Transcription Rate Analysis of Liver Slice Cultures Compared to Normal Liver and Monolayer Cultures.

Autoradiographs of plasmid-DNA containing filters prepared and hybridized as in Figure 1. Equal counts of nascent-labelled 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 in Materials and Methods and cultured for 24 hours under an atmosphere of 95% O_2 and 5% CO_2 with a total of five changes of culture medium each. The combined slices from three livers were split into three equal aliquots for B, C and a third culture (not shown, described in Table 9). The plasmid DNA probes used (Table 1 and 2) 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).

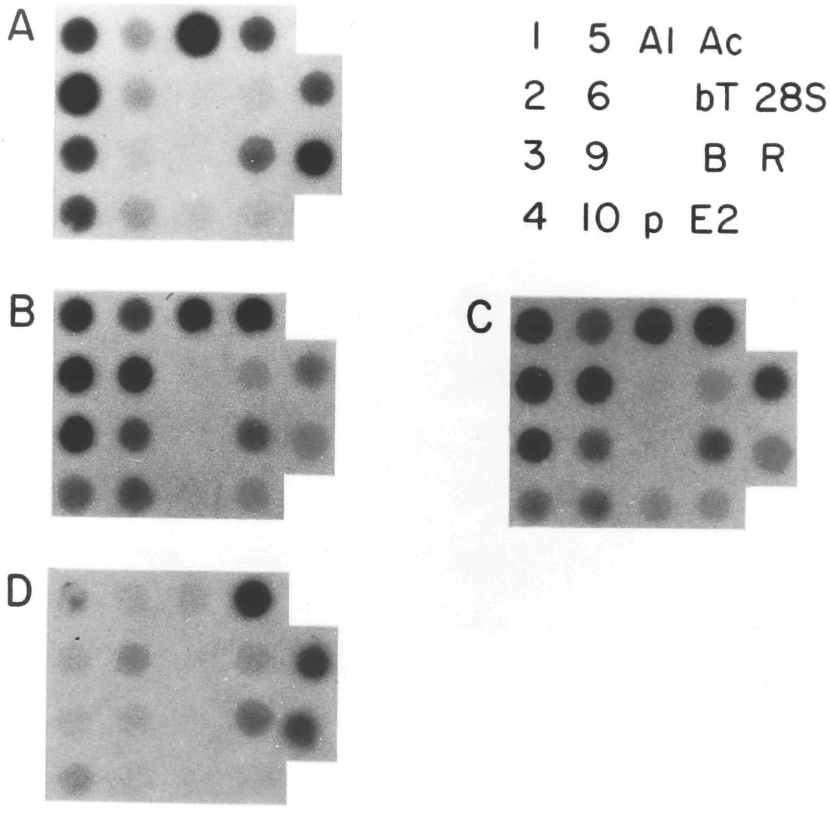

A
B
C
D
1 5 Al Ac
2 6 bT 28S
3 9 B R
4 10 p E2

Table 9. Densitometric Quantitation of Transcription Rate Analysis.

(liver = 100)

	Slice Culture	Slice, No Added Hormones	Slice, No Serum	Mono.
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
a1	22	19	23	0.2
ac	197	234	158	187
bT	150	151	148	150
CHO-B	100	100	100	100

Data from Figure 5 was analyzed by densitometric scanning, along with data from a parallel experiment in which an aliquot of the same slice preparation was cultured in basal culture medium lacking serum (this resulted in an approximate 50% decrease in yield of labeled nuclear RNA). 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 (Materials and Methods). Data for each transcription unit in the various cultures is presented in the table as a percentage of the signal measured in fresh mouse liver for that transcription unit.

Figure 6. Transcription in Stationary vs. Shaking Slice Cultures.

Autoradiographs of filters hybridized with nascent-labelled 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 slices prepared from two livers was cultured in basal medium as described in Figure 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 in air. Quantitation is presented in Table 10.

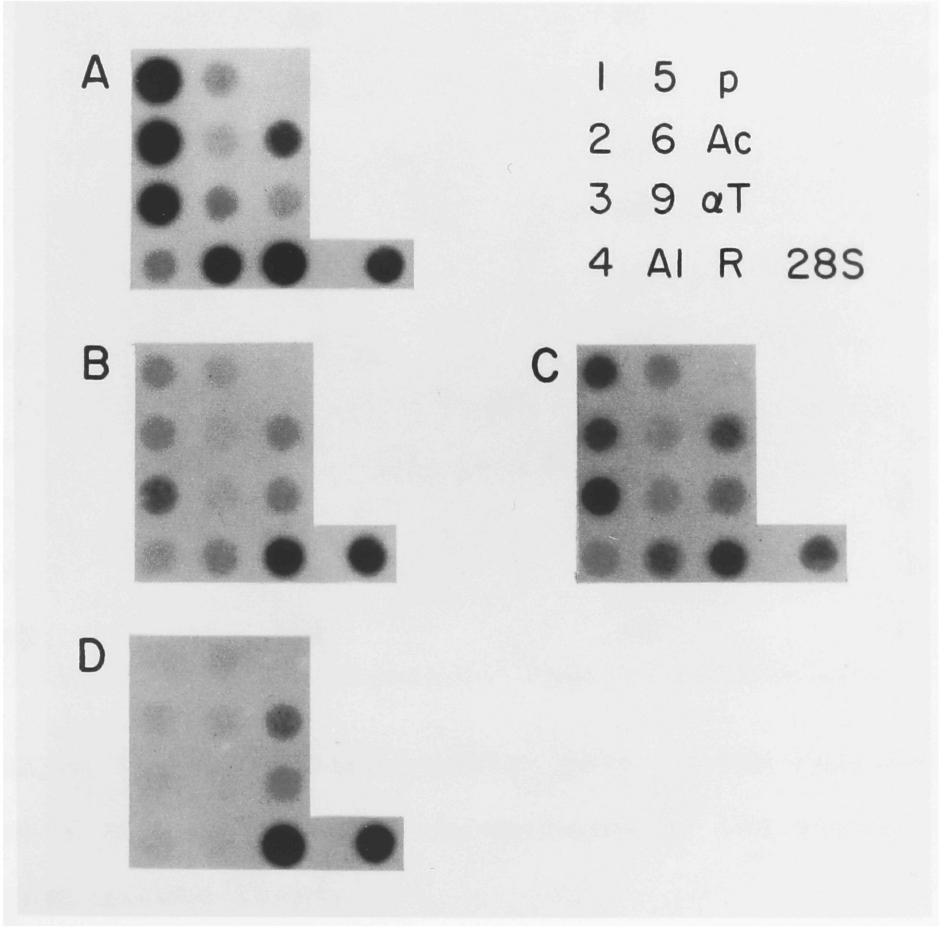

A
B
C
D
1 5 p
2 6 Ac
3 9 αT
4 Al R 28S

Table 10. Densitometric Quantitation of data in Figure 6.

(liver = 100)

	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
A1	13	25	2
Ac	28	53	46
aT	106	135	112
tR	33	24	64
28S	112	48	119

Values for each transcription unit in the cultured tissue and cells are expressed as percentages of the signal measured in fresh sliced liver.

cells cultured in monolayers. In contrast, transcription of common mRNA sequences (actin, 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 to fresh liver in the first experiment (Fig. 5, Table 9). This effect was not observed in the second experiment (Fig. 6, Table 10). 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. 6C) transcribed liver-specific genes at about twice the rate of slices suspended in shaking cultures (Fig. 6B, Table 10).

Also, 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.

Another way to view the gene-to-gene variations in the quantitative degree of change in transcription is to notice that transcription of the various liver-specific genes fell to a similar low level in the cultured hepatocytes. Genes that started with a higher rate of transcription had farther to fall before reaching this plateau. The quantitative significance of the low residual signals measured in monolayer cultures is unclear, because although they were usually greater than the background hybridization to plasmid vector DNA alone, they are statistically less reliable than more intense signals. 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 (Salditt-Georgieff, et al., 1984).

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. This is consistent with a similar observation reported in Chapter 3.

A final observation concerning the data in Fig. 5 and

Table 9 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. 5C, Table 9) resulted in only a slight decrease in liver-specific transcription. Omission of serum from the medium resulted in a decrease in total RNA synthesis, but had little or no effect on the relative pattern of tissue-specific transcription (Table 9).

IS LOST FUNCTION IN MONOLAYERS DUE TO CELL DAMAGE?

The experiments just described strongly suggest that liver cells require participation in an intact tissue in order for high levels of liver-specific transcription to be maintained. However, one remaining explanation for the loss of function in collagenase-dispersed cells exists: perhaps the cells have been damaged somehow by the process of perfusion, and in particular by the various proteolytic activities in the enzyme preparation. Therefore, a method for hepatocyte isolation that did not employ proteolytic enzymes was sought. Rat hepatocytes can be released from their normal tissue contacts by perfusion of the liver with EDTA (Berry, et al., 1983). Similarly, I found that perfusion of mouse livers for one hour with 2-10 mM EDTA in Hepes-buffered Hank's salts, followed by gentle teasing of the liver, resulted in a preparation of single cells (30-50% viable) and a large acellular mass, presumably the undigested reticular network and capsule. Viable cells were centrifuged through a

Percoll gradient, 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. 7), transcription of plivS-1 (MUP) was already greatly decreased, and transcription of actin was stimulated, just as with cells isolated with collagenase (Fig 2). After 24 hours in culture on plastic culture dishes, the EDTA-dispersed cells (Fig. 7C) showed the same specific transcriptional decline as cells isolated with collagenase, (Fig. 7D) suggesting that the action of collagenase was not the direct cause of the decline in tissue-specific transcription.

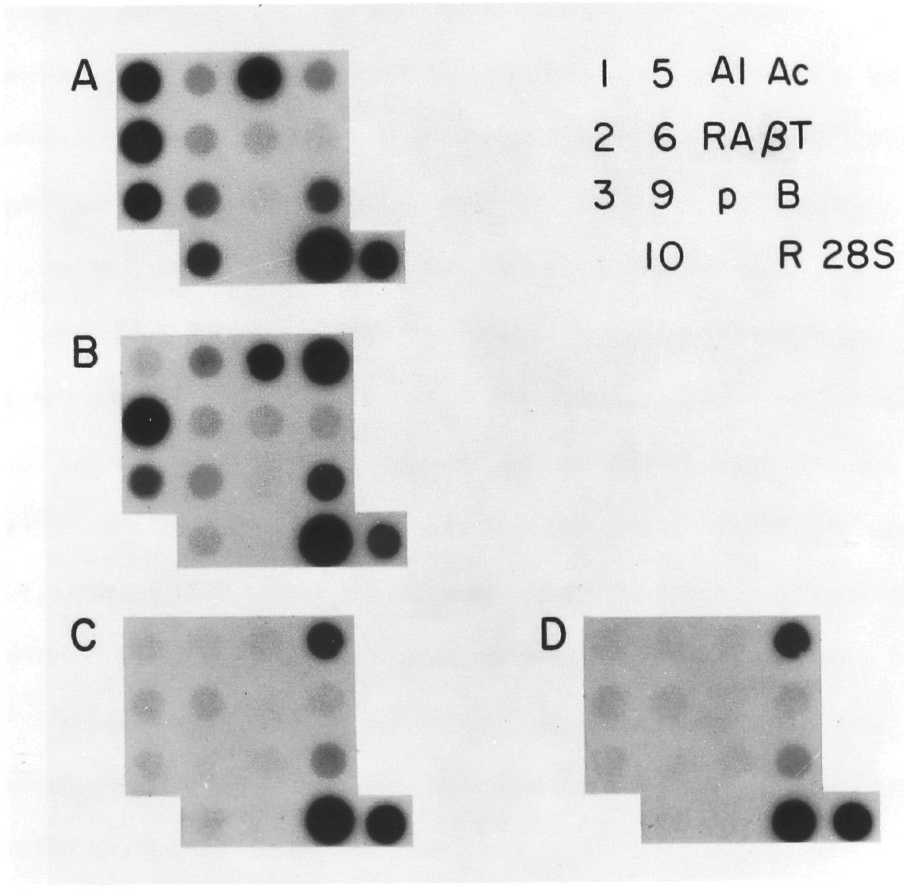
CAN TISSUE STRUCTURE BE RECONSTITUTED?

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 hepatocytes will form aggregates in suspension culture (McGuire and Burdick, 1976; Jeejeebhoy and Phillips, 1976), I have not found such aggregates to be stable or viable over the 24 hour period required to observe the change in transcription. Perhaps the reticular network of fibers surrounding cells in intact liver (Jones and Spring-Mills, 1983) is necessary to mediate cell interactions or stabilize the tissue structure.

However, I noticed when dispersing hepatocytes by EDTA

Figure 7. Transcription in Hepatocytes Dispersed with EDTA vs. 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 hours of culture (B) and 24 hours of culture (C), and from hepatocytes dispersed by the conventional collagenase perfusion technique followed by 24 hours of culture (D).


A
B
C
D
1 5 Al Ac
2 6 RA βT
3 9 p B
10 R 28S

perfusion (described above) that an acellular white mass (presumably the remnants of the undigested extracellular matrix) remained after the cells were disengaged. It seemed possible that the hepatocytes might "re-engage" within the liver structure after EDTA treatment if they were perfused with complete tissue culture medium. Therefore, after a one hour perfusion with the EDTA solution, perfusion was continued for another 30-80 minutes with the standard culture medium containing 20% fetal calf serum, normal divalent cation concentration, and no EDTA. To assay re-engagement, livers were sliced or the capsule was stripped and the released cells were counted. Data from such an experiment are shown in Table 11. At least 80% of hepatocytes were released from the liver after EDTA perfusion, while after another 50 minutes 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 these reengaged livers were somewhat smaller and more easily fragmented than slices of normal liver, they survived the same 24 hour culture period shaking in Erlenmeyer flasks. Nuclei isolated from these cultured reengaged slices were found to be transcribing liver-specific genes at rates similar to normal slices (Table 12), and well above the rate of transcription in nuclei from cells disengaged by EDTA but cultured as a monolayer.

Table 11. Cell Release Assay of Re-engagement.

PERFUSATE AND TREATMENT	# CELLS RELEASED X 10^{-6}	% VIABLE
HEH for 60 min. and tease	100	34
DMEM for 60 min. and tease	2	0
BCM + 15 mM EDTA, "	80	5
HEH for 60 min. and slice	80	24
HEH for 60 min., followed by BCM for 50 min. and slice	20	<5

Livers were perfused as indicated. Cells released into the medium by each treatment were counted and assayed for viability by trypan blue exclusion. HEH: Hank's salts, 10 mM EDTA, 5 mM Hepes, pH 7.3. DMEM: Dulbecco's modified essential medium; BCM: basal culture medium (containing serum and hormones). Flow rate in all cases was 6 ml/min, and otherwise perfusions were performed as described for the preparation of primary hepatocytes (Materials and Methods).

Table 12. Transcription in Re-engaged Liver Slice Cultures Compared to Fresh Sliced Liver, Normal Slice Cultures and Monolayer Cultures.

(liver = 100)

	Normal Slice	Reengaged Slice	Monolayer
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

Livers were disengaged by one hour perfusion with HEH (Figure 7, Table 11) to prepare monolayer cultures, or were then perfused for an additional one hour with basal culture medium (Table 11) to prepare reengaged slices. These cultures and standard slice preparations were exposed for a total of 24 hours to basal culture medium under 5% CO_2 in O_2 Transcription rate analysis was performed as described in Figure 5 and Table 9. Signals for each transcription unit are expressed as percentages of the signal in fresh slices of liver. The "less than" symbol (<) indicates the signal was no greater than the signal for non-specific binding of labeled RNA to pBR322 DNA on the same filter.

CONCLUSIONS

Transcription of most liver-specific but not common mRNA sequences declined sharply (ten to a hundred-fold) in cultured hepatocytes when they were disengaged from the normal tissue structure by either proteolytic or EDTA treatment. However, transcription continued at high levels (20 to 100% of normal) if the cultured, explanted cells were maintained in a tissue-like organization. Added hormones and serum made slices perform only slightly better than slices cultured without these additives. Furthermore, when slices were made and cultured for 24 hours from livers which had been disengaged with EDTA perfusion, but then immediately allowed to reengage by continued perfusion with a complete serum-containing medium, tissue-specific transcription continued at a high rate. Cells in reengaged tissue slices were exposed to the same solutions and the same stresses as cells separated by EDTA perfusion, with one exception: they quickly re-established extracellular contacts <u>in situ</u>, and this seems to be crucial for maintaining the normal transcriptional profile. These experiments imply 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.

CHAPTER SIX

LOSS AND RECOVERY OF A LOW LEVEL OF LIVER-SPECIFIC GENE EXPRESSION IN HEPATOMA CLONES

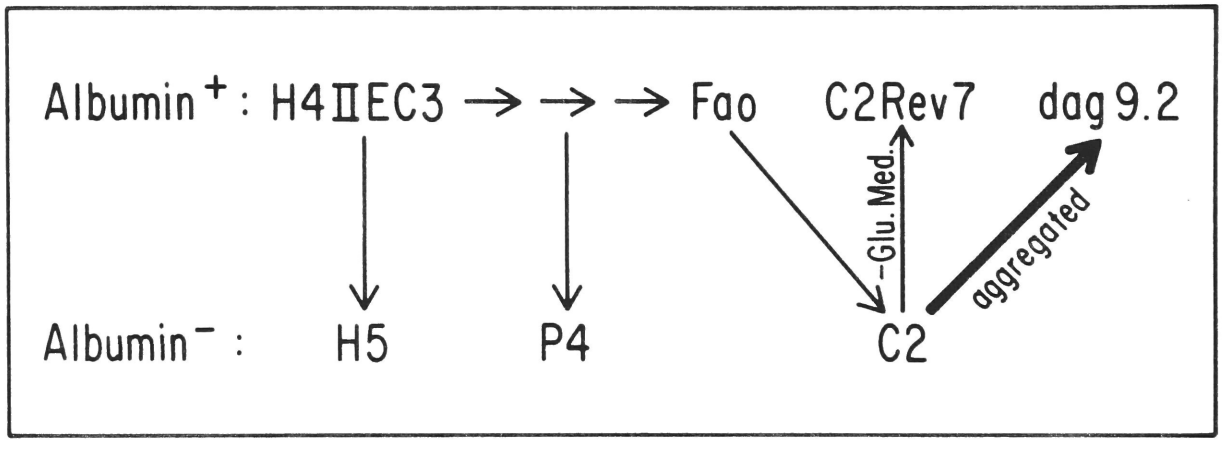
The experiments of the previous chapters have used physical techniques to perturb normal liver cells, and have shown that these cells require participation in a tissue structure for high levels of liver-specific transcription to proceed. Cells outside the tissue continued to display very low liver-specific transcriptional signals, but the physiological significance of these residual signals was uncertain.

This chapter describes experiments conducted with another type of liver (or liver-derived) cell maintained outside the normal tissue: a hepatoma cell. The experiments were originally begun with the objective of learning whether or not certain hepatoma cell lines, reported to maintain high levels of liver function, might not prove to be exceptions to the rule that cells outside the tissue have low levels of liver-specific transcription. In the course of the analysis, the power and potential - and limitations - of somatic cell genetic techniques for analyzing coordinate gene control became apparent. Information was also gained about the possible physiological significance and control of low-levels of liver-specific mRNA production.

Figure 8. Filiation of Rat Hepatoma Cell Lines Used in These Experiments.

Cell lines on the bottom row (C2, H5, P4) have negligible levels of expression of several enzymatic activities characteristic of liver cells, and secrete no detectable serum albumin. 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 following transient aggregation of C2 cells. Fuller discussion and references are presented in the text.

(Fig. 8)


Albumin+ : H4IIEC3
Fao
C2Rev7
dag 9.2
-Glu. Med.
aggregated
Albumin− :
H5
P4
C2

These experiments were conducted in collaboration with M.C. Weiss.

CHARACTERISTICS OF RAT HEPATOMA CELL LINES UNDER STUDY.

A family of rat hepatoma clones (see Figure 8) originally descended from the Reuber H35 hepatoma (Reuber, 1961; Pitot et al., 1964) has been established and studied extensively. As reviewed in the Introduction and summarized in Figure 8, the transition of these cells from a "differentiated" to an "undifferentiated" phenotype and back has suggested the existence of mechanisms that coordinate the expression of the tissue-specific functions they display. Furthermore, from cell hybridization experiments with these lines (Weiss, 1982; Killary and Fournier, 1984) has come evidence for diffusable factors involved in the regulation of specific liver functions. However, the quantitative level of liver function in these cells has never been compared directly with the level of function in normal liver cells. And although the coordinated changes in liver function observed in these cells have been assumed to arise through changes in transcription of specific genes, this point has never been directly demonstrated. In fact, with the exception of the albumin gene (Cassio, 1981), there was no direct evidence that changes in liver function in these cells necessarily involve changes in specific mRNA concentrations at all.

Therefore we undertook an analysis of liver-specific and

common mRNA production in the following cell lines, compared to normal cells in the rat liver. Clone Fao expresses numerous liver functions (Deschatrette and Weiss, 1974; Deschatrette et al., 1980) and is considered the "parental" cell type in these experiments. Clone C2 is a derivative of Fao in which liver functions including the ability to grow in glucose-free medium were spontaneously extinguished (Deschatrette, et al., 1980). C2Rev7 is a spontaneous variant cloned from C2, and was isolated by selection for cells which had recovered the ability to grow in glucose-free medium; other liver functions as well were found to be expressed in C2Rev7 (Deschatrette, et al., 1980). dag 9.2 is another cloned variant derived from C2: "dag" type cells are isolated at high frequency after C2 cells are allowed to aggregate transiently (Deschatrette, 1980). Although some liver functions are manifest in dag cells, they do not grow in the absence of glucose. Clones H5-6 and P4 are spontaneously occurring variants descended from the ancestral cell line which were selected on the basis of altered morphology, express none of the liver functions examined, and do not give rise to revertants to the differentiated phenotype at a detectable rate (Deschatrette, et al., 1980).

ANALYSIS OF SPECIFIC mRNA CONCENTRATIONS

In addition to the mouse cDNA probes used in other experiments described in this thesis, several rat cDNA probes (obtained from other laboratories, see Table 1) were used in

the analysis of these rat hepatoma cell lines. The liver-specific or liver-abundant mRNAs examined include those encoding three serum proteins produced by the liver (albumin, transferrin and alpha-1-antitrypsin), a principal enzyme in gluconeogenesis (phophoenolpyruvate carboxykinase, or PepCk), two other intracellular enzymes (phenylalanine hydroxylase and ligandin or glutathione-S-transferase), 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, alpha- and beta-tubulin) and two of unknown function but found in many cell types (CHO-A and CHO-B).

We began with a "Northern blot" analysis (Materials and Methods) to measure the relative concentration of the various mRNAs in the different hepatoma cell lines compared to normal rat liver. As shown in Figure 9 and Table 13, the cell lines divided cleanly into two sets with respect to the accumulation of liver-specific mRNAs. Cell lines which display liver functions (Fao, dag 9.2, and C2Rev7) possessed all eight liver-specific mRNAs measured. Cell lines in which liver functions have been lost (C2, H5 and P4) lacked these mRNAs with two exceptions: trace amounts of RNA hybridizing to the ligandin probe and low amounts of RNA hybridizing to the transferrin probe were detected in C2 and H5. The presence of transferrin mRNA in these two cell lines is interesting, since transferrin mRNA is similar or identical to the mRNA complementary to plivS-6 (Citron, Inouye, and

Figure 9. 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 Figure 12) as described in Materials and Methods. 25 mcg of each RNA was analyzed by the Northern blot procedure (Materials and Methods). The integrity of the RNA and the equivalence of inputs was verified by observing the ribosomal RNA bands in the ethidium bromide-stained gels under uv irradiation. Four identical blots were prepared, and were hybridized serially to three different probes; following each hybridization the probe was eluted by washing the blot in 0.1x SSC at 90°C for 30 minutes. 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 description of the plasmid probes used. Quantitative data is presented in Table 13.

Phe H'lase = phenylalanine hydroxylase.

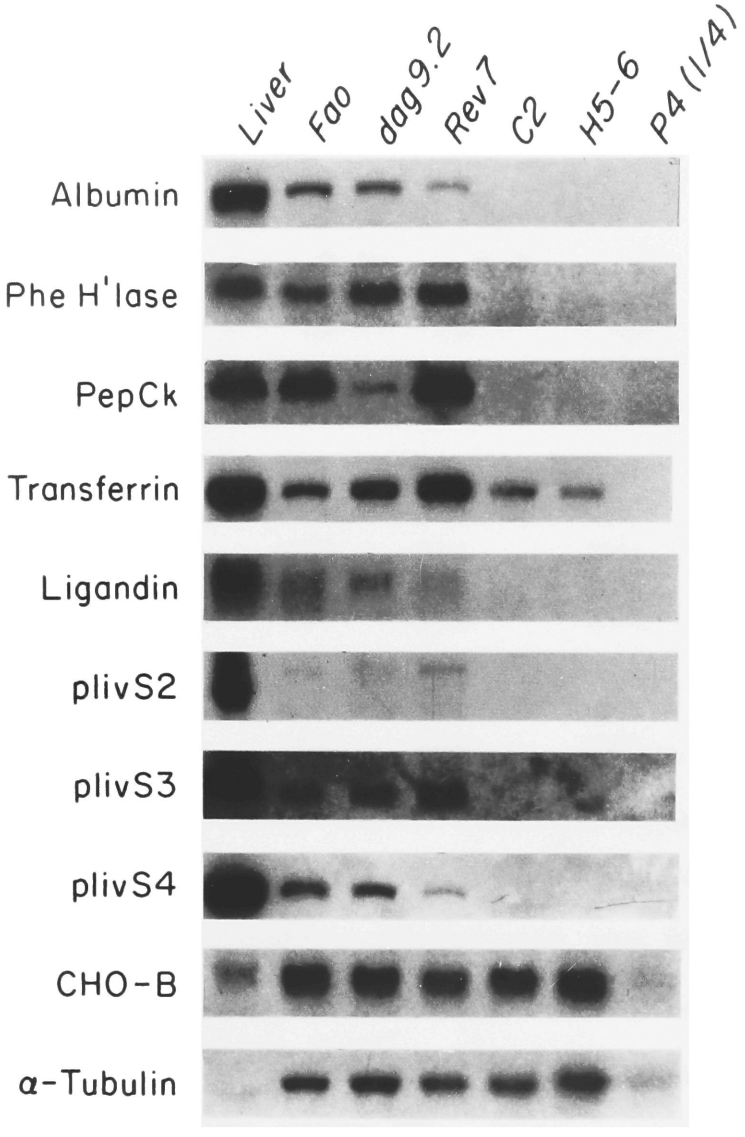

Liver
Fao
dag9.2
Rev7
C2
H5-6
P4 (1/4)
Albumin
Phe H'lase
PepCk
Transferrin
Ligandin
plivS2
plivS3
plivS4
CHO-B
α-Tubulin

Table 13. Relative Abundance of Various mRNAs in Hepatoma Clones Compared to Normal Liver.

(Liver = 100)	Fao	dag	C2Rev7	C2	H5	P4
Albumin	16	13	5	0	0	0
Phe 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	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
a-tubulin	1400	1680	1302	1456	2212	2968
b-tubulin	386	428	185	127	355	108

Autoradiographs from the experiment shown in Figure 9 were analyzed by densitometry (Materials and Methods). The value of each signal is expressed as a percentage of the corresponding signal in liver. Analysis using the S1 nuclease protection assay (M. Weiss, personal communication) confirmed the absence of albumin mRNA in C2, H5, and P4.

Darnell, unpublished observations), the only one of 11 mouse liver-specific mRNAs examined which was largely retained in hepatocytes when they were placed in primary culture (Fig. 2).

Although Fao and the two revertant cell lines dag 9.2 and C2Rev7 contained each of 8 liver-specific mRNAs, the amounts of the specific mRNAs varied compared to 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 was present at from 5 to 16% of the concentration in the liver. The RNA complementary to plivS-2, a liver specific mRNA that is made only in late fetal and adult hepatocytes (Powell, et al., 1984) is present at very low levels in all the hepatoma cell lines.

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

At a qualitative level, however, 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 that grows in glucose-free medium, not only has more than twice as much PepCK mRNA as liver cells, it also contains significant amounts of phenylalanine hydroxylase, albumin, transferrin, ligandin and

alpha-1-antitrypsin. Clearly the last four proteins are not required specifically for growth in glucose-free medium. The dag 9.2 cells were selected only for a changed morphology and not by metabolic slection, but have recovered all of the liver-specific mRNAs as well. These results strongly support the earlier suggestion (Deschatrette, et al., 1980) that general elements exist in the cell to coordinate liver-specific functions, and that these elements may be lost (or modified) and regained. These results further suggest that such an element may function by regulating specific mRNA concentrations.

ANALYSIS OF ALBUMIN GENE TRANSCRIPTION

To determine whether the observed variations in specific mRNA concentrations were the result of coordinated differences in the transcription of specific genes, the dot hybrid assay of transcription was employed. The initial focus was on transcription of the albumin gene. Four cloned segments of this gene were available (Fig. 10; Sargent et al., 1981) and were used to obtain an accurate profile of polymerase activity across the albumin transcription unit (Fig. 11). In addition to these segments, a subclone immediately 5' to the transcription initiation site was included, as well as control plasmids containing DNA complementary to beta-tubulin mRNA and tRNA-arginine. Fao and one of the revertants (C2Rev7) were compared to normal liver and three undifferentiated variant cell lines (clone II 5.0 is a revertable variant similar to C2).

Figure 10. Analysis of Albumin Gene Transcription in Rat Hepatoma Cells and Normal Liver: Map of Genomic Subclones Used.

A map of the rat albumin gene is shown, with exons represented by vertical bars. The extent of each genomic subclone is indicated by horizontal stripes above the gene. An alternative subclone nomenclature was used for clarity in this paper. The original, published names and insert sizes of the subclones are as follows (Sargent et al., 1981): JC (A10), 1 Kb; JB (A11), 1.2 Kb; C (A12), 2.4 Kb; B (A13), 1.7; D (A14) 1.0 Kb.

(Fig. 10)

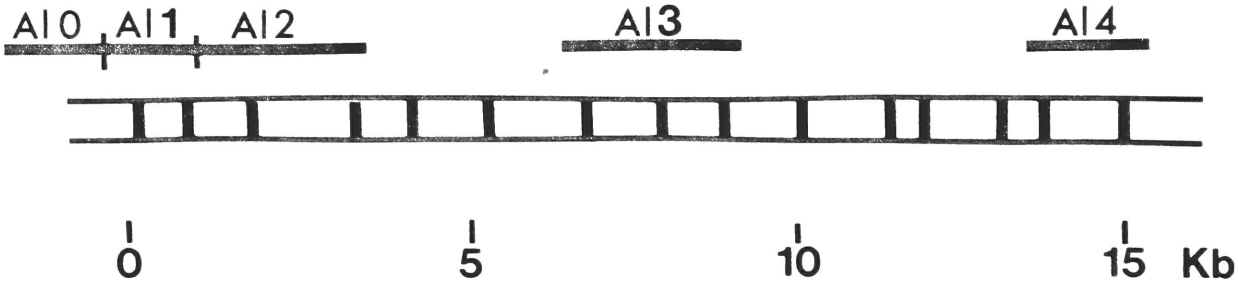
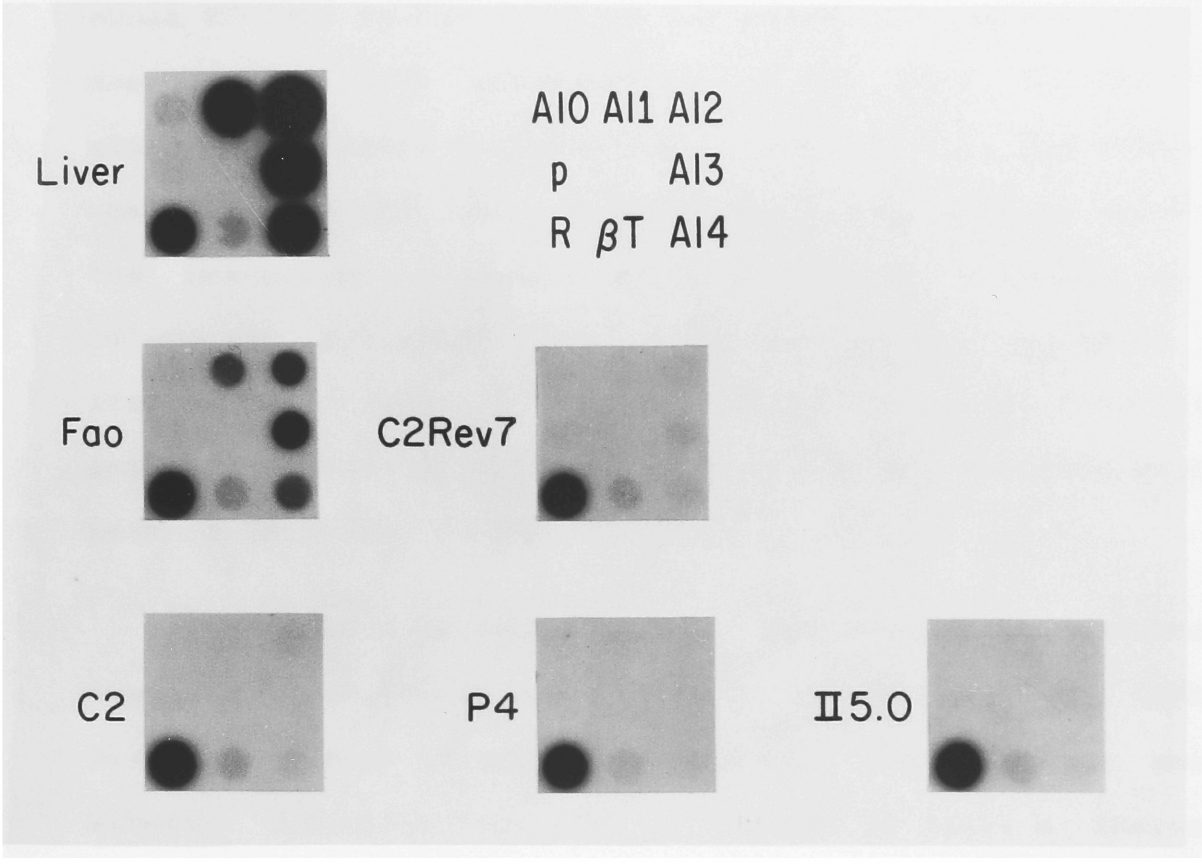


Figure 11. Dot Hybrid Analysis of Albumin Gene Transcription in Rat Hepatoma Cells and Normal Liver.

Equal amounts of nascent-labelled 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 (described in Figure 10 and Table 2). Autoradiographs of filters following hybridization are presented.


Liver
A10 A11 A12
p A13
R βT A14
Fao
C2Rev7
C2
P4
II5.0

Two surprising conclusions emerged from the results of this experiment (Fig. 11). First, even in the well differentiated clone, Fao, transcription across the albumin gene was much lower than in the normal liver. The average decrease in signal intensity for the four genomic segments (A11, A12, A13, and A14) was estimated by densitometry to be about 25-fold in Fao relative to liver. This contrasts with a decrease of only about 6-fold in the mRNA concentration observed in Figure 9 (Table 13). Little or no hybridization was obtained with the DNA upstream of the cap site (A1-0) 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 transcription of the entire transcription unit in both cell nuclei.

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, albumin transcription must be reduced at least a thousand-fold in C2Rev7 compared to normal hepatocytes. Yet the albumin mRNA concentration is only reduced about 20 fold in this cell line (Table 13). Cell lines which contained no detectable albumin mRNA (C2 and H5) did not transcribe the gene at detectable levels.

TRANSCRIPTION ANALYSIS OF OTHER GENES.

Thus it appeared that mechanisms for selective stabilization or efficient processing of mRNA were combined with mechanisms of transcriptional control to determine the albumin mRNA concentrations in these cells. To confirm and extend these results, the transcription assay was repeated with a broader range of plasmid probes, including most of those whose relative mRNA concentrations were determined in Figure 9 and Table 13. The nuclei analyzed in this experiment were taken from the same rat liver and cultures as were used in the analysis of mRNA concentrations (Figure 9).

The results (Fig. 12, Table 14) reproduced the previous result for albumin gene transcription and in addition gave evidence of apparent post-transcriptional modulation of other tissue-specific mRNAs as well. First, in the differentiated cultured cells (Fao, dag, C2Rev7) disproportionately low transcription rates relative to mRNA concentrations were observed for all five liver-specific mRNAs assayed (albumin, PH, PepCk, Transferrin and Ligandin). Nuclei from Fao cells, for example, transcribed these mRNAs from about 2.8 to about 7.8 times more slowly than would have been expected from their relative mRNA concentrations (Table 15). In contrast, two common mRNAs (CHO-A and CHO-B) showed little or no increase in stability or efficient processing in the cultured cells. The actin and tubulin mRNA concentrations also increased apparently through post-transcriptional mechanisms, similar to observations made earlier in this thesis.

Figure 12. Transcription Rate Analysis of Various Genes in Rat Hepatoma Clones and Liver.

Experimental procedure is the same as in Figure 11. Plasmids are described in Tables 1 and 2 and Figure 10. Quantitation is given in Table 14.

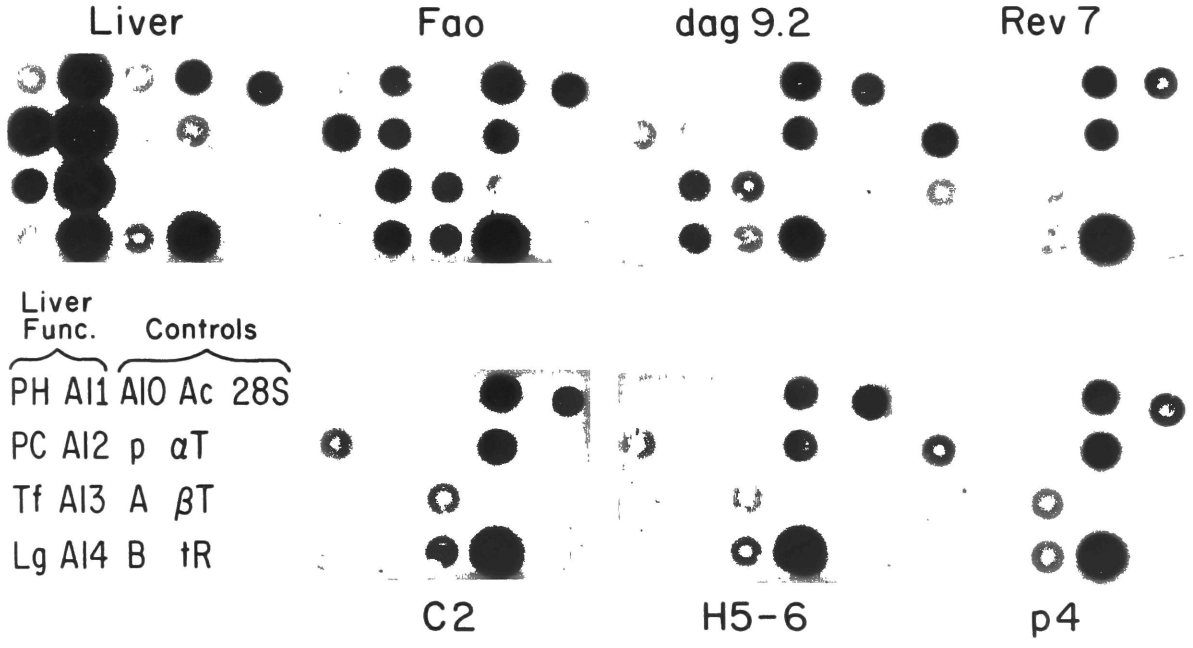

Liver
Fao
dag 9.2
Rev 7
Liver Func.
Controls
PH AI1 AI0 Ac 28S
PC AI2 p αT
Tf AI3 A βT
Lg AI4 B tR
C2
H5-6
p4

Table 14. Relative Transcription Rates of Various mRNAs in Hepatoma Clones Compared to Normal Liver.

(Liver = 100)	Fao	dag	C2Rev7	C2	H5	P4
Albumin						
A1-1	2.8	0.6	0.03	<0.03	<0.03	<0.03
A1-2	2.8	0.8	<0.1	<0.1	<0.1	<0.1
A1-3	7.8	3.5	<0.2	<0.2	<0.2	<0.2
A1-4	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
Phe hydroxylase	33.8	51	106	38	21	30
PepCK	22.7	2.5	18.4	3	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	1400	1246	616	980	644	1148
CHO-B	200	98	114	190	134	166
b-actin	262	254	141	220	66	157
a-tubulin	283	223	258	266	201	552
b-tubulin	136	78	231	101	83	148
tRNA-arginine	117	63	118	131	130	98
28S rRNA	100	72	88	47	138	87

Densitometric quantitation (Materials and Methods) of data in Figure 12 is presented. The value for each signal, after subtraction of the value for the pBR322 signal on the same filter (non-specific background), is expressed as a percentage of the corresponding signal in rat liver. Where a signal is no greater than the signal for pBR322 in the same hybridization, the "less than" symbol (<) is included in the table.

Table 15. Ratios of Abundance and Transcription Rate for Selected mRNAs in Hepatoma Cell Lines.

	Concentration/Transcription			
	Fao	C2	dag	C2Rev7
albumin	3.6	0/0	7.2	>56
phe 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
a-tubulin	4.9	5.5	7.5	5.0

Data from Tables 13 and 14 are expressed as mRNA Concentration divided by Transcription Rate. A number of greater than 1.0 (0.6 - 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 transcriptional analysis of the undifferentiated variants (C2, H5-6, P4) provided two apparent examples of post-transcriptional repression of mRNA accumulation. The mRNAs for PepCk and phenylalanine hydroxylase were undetectable in these variant cell lines (Figure 9), yet substantial transcription signals for these mRNAs were obtained in each cell line (Figure 12). For phenylalanine hydroxylase the transcription signals were essentially unchanged compared to the differentiated cells, and for PepCk they were reduced 5 to 10 fold relative to Fao and C2Rev7 but were equivalent or greater than than the transcriptional signal in dag 9.2 cells. These results imply a post-transcriptional failure either in RNA processing or stabilization in the undifferentiated cells. The present data are based on hybridization to a single cDNA clone, however, and the possibility remains that the residual transcriptional signal generated in the de-differentiated cells did not result from transcription of the entire PH or Pepck genes. It is even possible that some RNA other than PH or PepCk primary transcript may 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 using subclones across the gene (as was done for albumin, Figs. 10 and 11) 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 apparent post-transcriptional control of albumin, PepCk and phenylalanine hydroxylase should be considered in contrast to several examples of at least a limited degree of transcriptional regulation of some liver-specific mRNAs in the various cell lines. For example, the recovery of albumin mRNA in the revertant, dag 9.2, to a level similar to Fao (Fig. 9) coincided with a proportionate recovery of albumin transcription (Table 14 and 15). Also, variations among the differentiated cell types in the abundance of the tissue-specific mRNAs were observed (Table 13), and except for albumin in C2Rev7, most of these variations were consistent with proportional variations in transcription rate. For example phenylalanine hydroxylase mRNA is highest in C2Rev7 and this line also gave the highest transcription signal for PepCk. PepCk mRNA is high in both Fao and C2Rev7 and the transcription rate was highest for 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 TO MOUSE LIVER AND CULTURED HEPATOCTYES

Despite its "well-differentiated" phenotype, the transcription rate of tissue-specific genes in Fao is sharply reduced compared to normal liver (Figures 11 and 12). We

wondered whether this reduction might be a general property displayed by other hepatoma cell lines, and perhaps by all cells cultured outside the normal tissue structure, as suggested by the results of the previous chapter. A similar transcriptional analysis was therefore applied to the mouse hepatoma line BW1-J (Cassio and Weiss, 1979), known to express several liver functions. Normal mouse liver and primary mouse hepatocytes after one day in culture were compared in the same assay, which included the various mouse liver-specific cDNA clones described earlier.

Nuclei from BW1-J, like Fao, transcribed all tissue-specific mRNAs tested at a greatly reduced rate compared to normal mouse liver (Figure 13 and Table 16). The transcription rate for most liver-specific genes was not even as high as in nuclei from primary cultures of hepatocytes in this experiment, although the signals for albumin and transferrin mRNAs were clearly above background.

Figure 13. Transcription Rate Analysis of Various Genes in Mouse Hepatoma Clone BW1-J Compared to Mouse Liver and Primary Cultured Mouse Hepatocytes.

Primary cultures of mouse hepatocytes were prepared and cultured for 24 hours as described in Materials and Methods. Liver-specific gene transcription rates in such cultures decline sharply in the first 24 hours of culture and stabilize after 24-48 hours (Chapter 3). The same procedure as described in Figure 11 was used to analyze nuclei from mouse liver, cultured BW1-J cells and the primary hepatocytes. Plasmid DNAs used are indicated on the key, and described in Tables 1 and 2; quantitation is presented in Table 16.

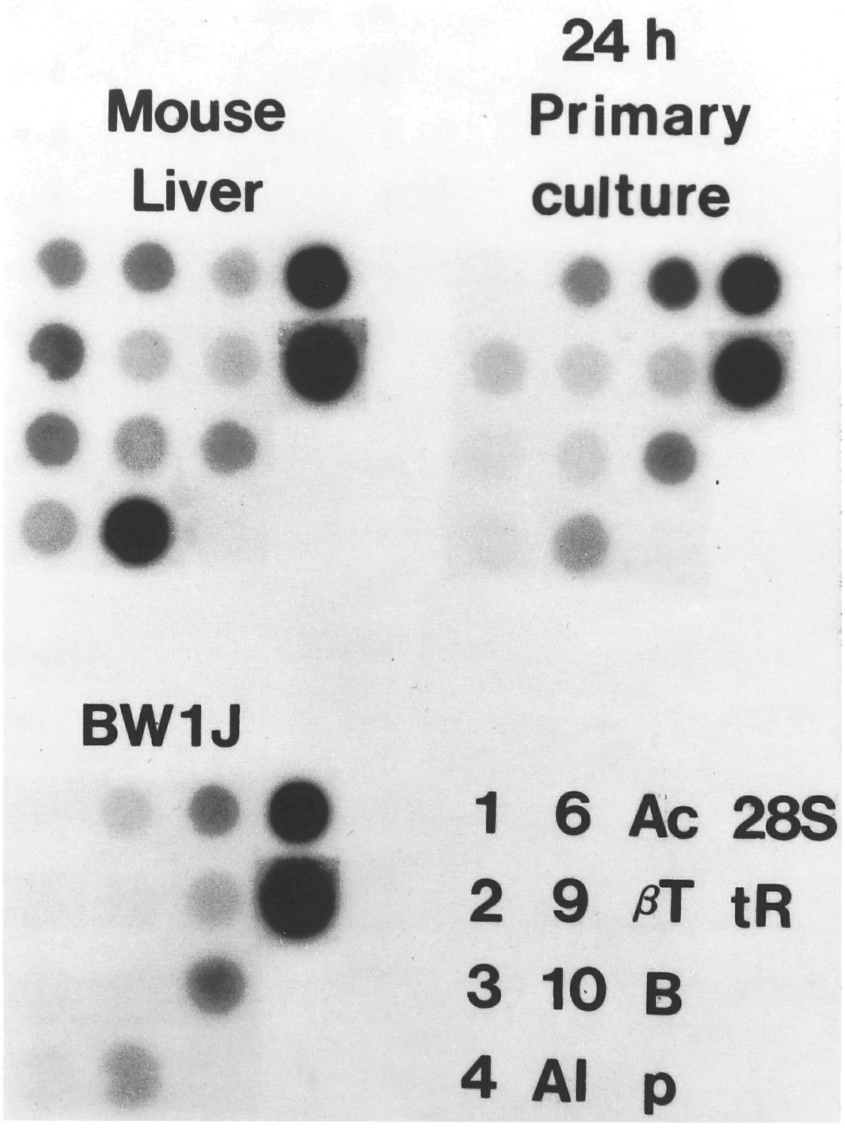

Mouse
Liver
24 h
Primary
culture
BW1J
1 6 Ac 28S
2 9 βT tR
3 10 B
4 Al p

Table 16. Quantitation of Data in Figure 13.

(Mouse Liver = 100)

	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
b-tubulin	115	105
CHO-B	147	150
28S rRNA	88	96
tRNA-arg	75	108

Densitometric analysis was applied as in Table 13.

Similar to the behavior of normal hepatocytes maintained outside the tissue, the isolated cultured hepatoma cells examined here also displayed low levels of liver-specific transcription. Nonetheless, the differentiated clones did contain detectable amounts of every one of eight liver-specific mRNAs assayed, showing that the low rates of liver-specific transcription (at least for these hepatoma cells) are physiologically significant. Furthermore, loss of differentiated function was accompanied by loss or large declines in all but one liver-specific mRNA (that encoding transferrin, which is also conserved in primary cultures). Recovery of one liver-specific function (e.g., gluconeogenesis) was accompanied by recovery of some amount of all liver-specific mRNAs examined. Thus these hepatoma cells may serve as appropriate models for the study of the underlying concerted activation of tissue-specific genes, but not for the processes which ultimately result in a high level of tissue-specific transcription in the liver.

Comparison of the mRNA concentration data (Table 13) and the transcription rate data (Table 14) suggests that liver-specific genes may be regulated at both transcriptional and post-transcriptional levels in these cells. Indeed, different cells may regulate the same mRNA at different levels to achieve a similar phenotypic result. This point is illustrated more clearly in Table 15, where the ratio of mRNA concentration to transcription rate has been calculated for

selected genes. Although albumin is transcriptionally repressed in C2, and its reactivation in dag 9.2 to a level equivalent to Fao appears to be transcriptionally mediated, the reapparance of albumin mRNA in C2Rev7 must involve significant increases in post-transcriptional stabilization or processing efficiency. This is reminiscent of the increase in albumin mRNA accumulation observed in normal hepatocytes cultured under hormonally-defined conditions (Chapter 4). It thus appears that different hepatoma clones may regulate the same gene differently.

Also consider the recovery of differentiated traits in dag 9.2 compared to its parent, C2 (Table 15). Albumin transcription is increased, and PepCk and PH transcription are apparently not, but all three mRNAs are now accumulated in dag cells (Figure 9, Table 13). It appears as if the cell may be able to regulate a set of genes by coordinating both transcriptional and post-transcriptional mechanisms to yield a general phenotypic result: accumulation (or loss) of many liver-specific mRNAs.

We also found evidence for a generalized increase at the post-transcriptional level in the efficiency of tissue-specific mRNA production in all of the differentiated clones compared to normal liver (Table 15). Without examining a much broader range of control genes, however, we cannot conclude with certainty that this effect is restricted to tissue-specific mRNAs. One simple possibility is that cultured

cells for some reason contain higher amounts of mRNA as a percentage of total RNA than do cells in the liver. Such a generalized increase in post-transcriptional efficiency of mRNA production must not be a general property of cells in culture, however, since cultured mouse hepatocytes do not seem to maintain similar amounts of liver-specific mRNAs from a transcriptional output similar to the hepatomas (Fig. 2).

CHAPTER SEVEN

SUMMARY, DISCUSSION AND CONCLUSIONS: A TWO-STEP MODEL OF LIVER-SPECIFIC GENE ACTIVATION

This final chapter begins with a summary of the experimental results presented in this thesis. Next, some of the broader implications of these findings will be considered. Finally, a simple two-step model for the activation of liver genes will be presented, and experimental tests of the model proposed.

SUMMARY OF RESULTS

When the liver is disaggregated and the isolated cells cultured under a wide variety of conditions, transcription of most liver-specific but not common genes declines sharply within 24 hours. However, cells exposed to the same culture conditions but maintained in slices of normal tissue do not show this large differential decline in tissue-specific transcription. Furthermore, the tissue may be reversibly "disengaged" and "reengaged," and cultured slices from livers so treated also maintain high rates of liver-specific transcription for at least 24 hours.

Regardless of the method of cell culture, transcription of common genes continues at fairly constant rates, although a large and transient increase in the transcription of actin mRNA sequences is noted in cells shortly after tissue

disaggregation. Concentrations of actin, tubulin and perhaps other common mRNAs increase apparently through post-transcriptional means in primary cultured cells. Albumin (and to a lesser degree a-1-antitrypsin) mRNA concentrations may also be selectively conserved through post-transcriptional mechanisms in primary cultures of normal rat hepatocytes grown in the appropriate medium.

Like isolated primary cultured hepatocytes, cultured "differentiated" hepatoma cell lines also transcribe liver-specific genes at relatively low rates. However, liver-specific transcripts are accumulated to higher concentrations relative to their rates of transcription in these hepatoma cells than in normal liver cells. The loss and recovery of liver-specific functions in "undifferentiated" variant hepatoma cell lines and "revertants," respectively, involve the coordinated loss and recovery of many different liver-specific mRNAs. Post-transcriptional regulatory mechanisms may play a significant role in these changes however.

RELATIONSHIP OF TISSUE STRUCTURE AND LIVER-SPECIFIC TRANSCRIPTION IN DEVELOPMENT

These results show a clear relationship between tissue structure and high rates of tissue-specific transcription in adult liver cells. In fact, such a relationship may operate in the development of the liver as well. It has been noted that a number of liver-specific mRNAs in rodents increase in

concentration in the last quarter of fetal development (Barth et al., 1982; Muglia and Locker, 1984; Powell et al., 1984) at a time when cell-cell contacts and other structural aspects of the adult liver are formed and mature (Doljanski, 1960; Wood, 1965; Sasse, 1969; Montesano et al., 1975). Much of this increase appears to be due to increased rates of transcription of these genes (Powell et al., 1984). The possible impact on these measurements of changing proportions of hepatocytes and hematopoietic cells in the fetal liver (Silini, et al., 1967; Paul et al., 1969) must be considered, but there is no evidence that these changes in cell population could acount for the observed changes in tissue-specific mRNA concentrations, which approach or exceed a factor of 10. Thus it seems possible that liver-specific transcription in the fetal liver increases as the mature structure of the liver is gradually formed. Quantitative experiments using in situ hybridization and transcription rate measurements will be useful in further analysis of this phenomenon.

ROLE OF HORMONES IN MAINTAINING LIVER-SPECIFIC TRANSCRIPTION

Addition of a wide range of hormones and other soluble factors had no effect on the low rates of liver-specific transcription in isolated hepatocytes. Conversely, removing additional hormones or serum from the culture medium had minimal effect on the high rates of transcription in liver slices. This implies that hormones and other soluble factors

may play only a minor role in the maintenance of tissue-specific transcription in hepatocytes. However, these experiments do not measure the possible hormonal requirements for longer-term maintenance of cellular integrity and function, or for functions regulated at a post-transcriptional level (Jefferson, et al., 1984). Furthermore, these experiments do not address the possible role of hormones in signaling the onset of critical developmental processes (Greengard 1969, 1975). Finally, genes known to have a strong dependence on specific hormones (e.g., gluconeogenic enzymes; Granner and Hargrove, 1983) have not been specifically studied here. Several hormones including both androgens and glucocorticoids have a role in controlling major urinary protein production in the animal (Knopf, et al., 1983), but supply of these hormones did not maintain transcription of this gene (plivS-1) in cultured cells. And even with this gene, consistently higher levels of transcription were obtained in slices than in cells.

SIGNIFICANCE OF LOW LEVEL TRANSCRIPTION SIGNALS

Despite the dramatic drop in liver-specific transcription in isolated cells, very low but stable transcription of tissue-specific genes did appear to continue in hepatocytes outside the normal tissue organization, even after 10 days in culture (Table 6). The significance of these low level signals has been difficult to assess, for two reasons: primary cultures of hepatocytes are neither stable

nor homogeneous, and similar low level transcription signals for liver-specific genes have been detected on occasion in non-liver cells (unpublished observations, Powell et al., 1984). The experiments with hepatoma cells begin to address this issue, by showing that low liver-specific transcription rates in isolated cells can indeed result in the production of mRNA. Furthermore, other cell lines that produce undetectable amounts of these mRNAs can be derived, concomitant with a further decrease in the transcription rate of at least some of these genes. The possibility remains, however, that post-transcriptional mechanisms may have a significant role in the regulation of low transcriptional outputs (below).

POST-TRANSCRIPTIONAL CONTROLS

At first glance, the apparent involvement of post-transcriptional gene activation and repression mechanisms in the regulation of at least three and possibly all of the five liver-specific mRNAs whose transcription was examined in the rat hepatoma 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 (Derman, et al., 1981). However, even the highest rates of transcription measured in the differentiated hepatoma cells are so low that they would have fallen near or below the limits of detection in that study of liver-specific mRNA production. In fact, a hepatoma cell line similar to

the differentiated clones used was examined in the earlier study (Derman et al., 1981) and was scored as essentially negative for the transcription of most liver-specific mRNAs in those experiments. Only with the increased sensitivity of the autoradiographic dot hybrid assay was it possible to monitor low rates of transcription. The earlier study thus should be taken as demonstrating that maximal activation of liver-specific mRNA production is transcriptionally mediated, a result consistent with the response of normal liver cells to tissue disaggregation. Whether or not post-transcriptional controls influence or even predominate over low levels of transcriptional output remains a question for further study.

TWO STEPS IN LIVER-SPECIFIC GENE ACTIVATION

Based on these results, I suggest that the complete and concerted activation of tissue-specific genes in liver cells may actually require (at least) two steps: first the establishment of a basal level of mRNA synthesis and accumulation, and second, an increase in polymerase II activity on specific genes that is somehow dependent on the assumption of the mature tissue structure (Fig. 14). The first step might logically be expected to occur during embryonic induction, to result in cells capable of producing low levels of liver-specific mRNAs, as suggested above for fetal hepatocytes. The second step may depend on the generation of a signal through the tissue structure itself, for full differentiated function and maximum tissue-specific

Figure 14. Two Step Activation of Liver-Specific Genes.

A developmental model is outlined, based on information presented in this thesis.

PRE-LIVER: cells in the pre-hepatic endoderm immediately prior to hepatic induction by pre-cardiac mesoderm. Functionally equivalent to C2 hepatoma cells, which also do not express liver-specific functions but can give rise to differentiated cells at a high rate in response to an appropriate induction.

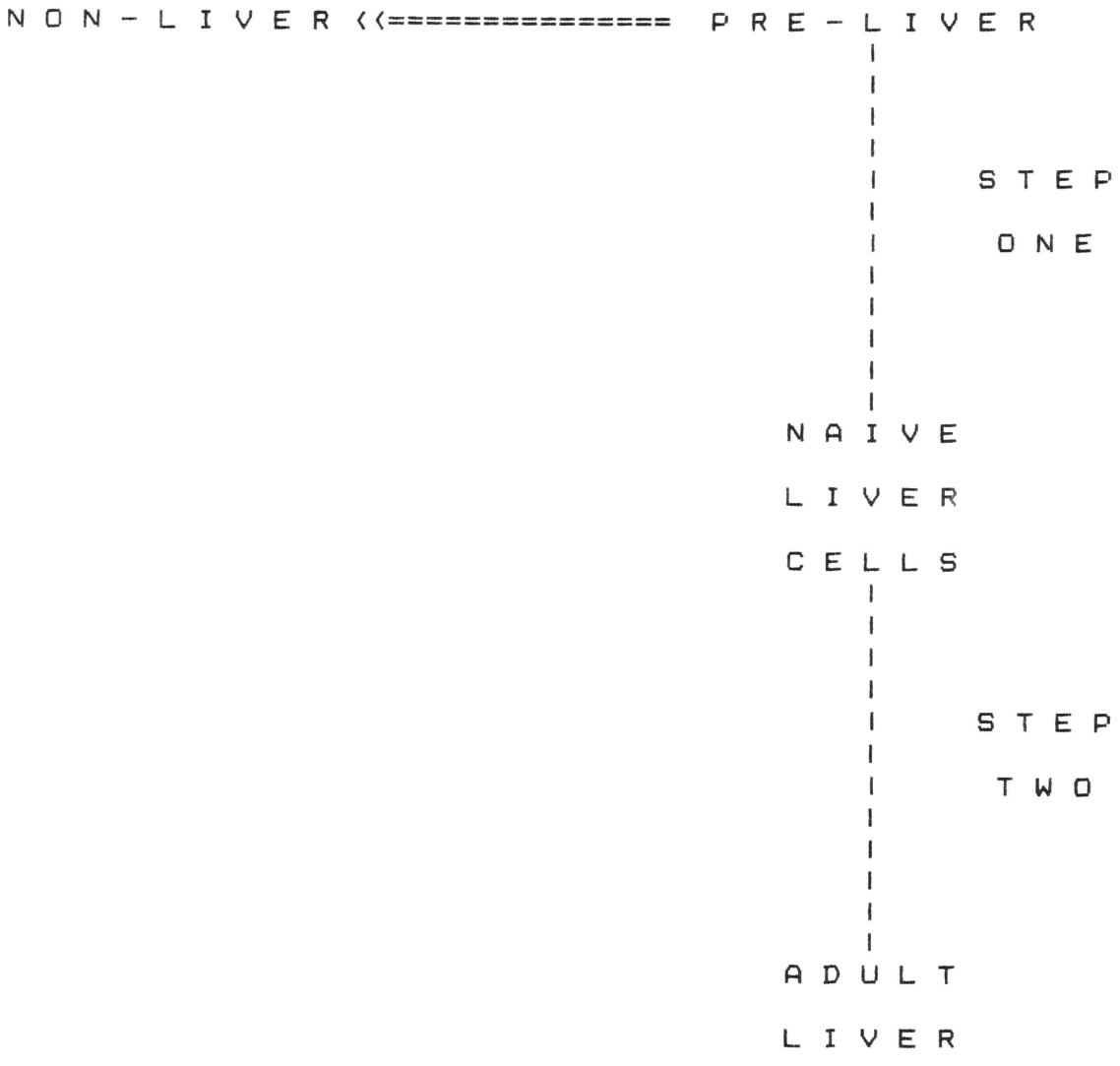
NAIVE LIVER CELLS: Cells isolated from or never exposed to the adult tissue environment. Included are primary cultured hepatocytes, differentiated hepatoma clones (e.g., Fao), and cells in the fetal liver. These cells produce liver-specific mRNAs but at a low rate.

ADULT LIVER: Cells in the adult liver produce liver-specific mRNAs at a high rate based on transcriptional amplification.

NON-LIVER: Once a critical window in development has closed, non-liver cells are no longer capable of expressing liver-specific genes, or giving rise to liver-like derivative cells at a detectable rate. Presumably genes not expressed at the time the developmental window closes are irreversibly inactivated (e.g., gene methylation).

STEP 1: competence for liver-specific mRNA production established through transcriptional and/or post-transcriptional mechanisms. (reversible).

STEP 2: amplification of liver-specific gene transcription rate dependent upon mature tissue structure. (reversible).


NON-LIVER ((========== PRE-LIVER
STEP ONE
NAIVE LIVER CELLS
STEP TWO
ADULT LIVER

transcription. When that signal is interrupted by tissue disaggregation, the level of tissue-specific transcription declines to the basal level established by the first mechanism.

WHY TWO STEPS?

There are at least two obvious biological reasons for having a two-step mechanism of liver-specific gene activation. First, efficiency: the liver in the fetus need not be required to function at maximal levels since maternal organs carry much of the biological burden. Second, evolution: it seems reasonable to suppose that organisms with a simple all-or-none distribution of functions among different cell types would have first arisen, followed by selection for organisms which had evolved secondary mechanisms to amplify or enhance tissue-specific functions at appropriate times or places. Furthermore, a mechanism of transcriptional amplification dependent upon cell and tissue structure seems appropriate for higher eucaryotic cells, given their structural complexity and the diversity of tissue-specific structural components like intermediate filaments (Lazarides, 1980).

MECHANISMS: COMPETENCE FOR LIVER-SPECIFIC mRNA PRODUCTION

The hepatoma cell lines examined in this work provide a convenient model system for studying at least some of the processes involved in establishing the underlying competence

for liver-specific mRNA production. Experiments using somatic cell genetic approaches over the last 15 years have provided a number of clues about the possible mechanisms. For example, the "reversion" of "de-differentiated" hepatomas to the differentiated phenotype is apparently indifferent to chemical mutagenesis, and some "de-differentiated" lines appear totally incapable of giving rise to revertants (Deschatrette et al., 1980). Unstable variants sometimes arise which temporarily express a subset of liver functions before gradually conforming to either the fully differentiated or de-differentiated phenotype (Moore and Weiss, 1982). When an "un-differentiated" or "de-differentiated" cell type is fused with a well-differentiated hepatoma cell type, at least some (and possibly all or many) liver functions are extinguished in the resulting hybrid (Weiss, 1982). In at least once case, a single chromosome has been identified that appears to contain a "repressor" activity for a single liver-specific function (Killary and Fournier, 1984).

The simplest hypothesis consistent with all these observations is the following: a positive diffusable activator of liver function supports the expression of liver-specific characteristics in these cells. This activator can be permanently inactivated or lost through mutation. The activator exists in a critical balance in the cell, perhaps through autocatalytic positive regulation. When activity of the activator falls below the critical threshold "de-

differentiation" occurs. When the activator activity is close to the threshold, phenotypic instability results. The activator is diluted or the cycle somehow disrupted by the process of cell hybridization. "Reversion" occurs stochastically, in a process insensitive to mutation: when random fluctuations of activator activity exceed the critical threshold the autocatalytic process is renewed and a "differentiated" clone arises. In addition to this positive regulator, other repressors or modulators of specific genes exist (e.g., Killary and Fournier, 1984), that may have arisen to fine-tune or segregate the expression of genes originally common to several tissues (or to a single evolutionary parent tissue).

An implication of this hypothesis is that it may be possible to introduce such an activator, its gene, or its modifiers into de-differentiated cells in such a way that the recipient should "revert" at a high rate. Such a strategy might allow eventual isolation of the activator gene or other components of the regulatory mechanism involved in establishing competence for liver-specific mRNA production. Retroviral vectors (Cepko et al., 1984; Sorge et al., 1984) could be used to introduce a library of sequences under the control of a constitutive viral promoter into de-differentiated hepatoma cells. Revertant cells could then be isolated based on the gluconeogenic selection scheme (Bertolotti, 1977a, 1977b; Deschatrette, et al. 1980), a selection procedure that has a low rate of leakiness and that

requires the simultaneous recovery of at least two independent tissue-specific functions.

Two areas of ambiguity concerning the behavior of these hepatoma cell lines should be noted: first, the biochemical level of regulation (transcription and post-transcription) involved in the loss and recovery of liver-specific functions in these cells is not obvious and may not even be unitary, as indicated by the results of Chapter 6. Second, rather complex hybridization experiments conducted with these cells have yielded rather complex results, that are difficult to explain by any simple hypothesis (Mevel-Ninio and Weiss, 1981; M. Weiss, personal communication).

The behavior of "de-differentiated" C2 cells suggests another intriguing parallel with normal cells in the developing embryo. C2 cells give rise to "revertants" at a high rate when allowed to aggregate transiently (Deschatrette, 1980). Hepatocytes are first produced in the embryo following a specific induction that requires cell-cell contact (Houssaint, 1980; Le Dourin, 1975). Whether or not the mechanisms involved in these two inductions are similar or even related remains to be investigated.

SIGNALS FOR DIFFERENTIATION: SPECIFIC MOLECULES VS. CRITICAL STATES

What sort of signals inherent in a mature tissue structure might underlie a tissue structure-dependent

amplification of tissue-specific transcription rates? One possibility is that the cell must receive a specific signal from neighboring cells or extracellular matrix through receptors at the cell surface. Although this possibility has not been eliminated, I have also obtained no evidence directly supporting it. For example, little or no change in tissue-specific transcription was observed in hepatocytes co-cultured with other liver cell types, or when seeded at different densities, or when cultured on extracellular matrix preparations (Table 6). Some of these culture techniques, of course, may result in enhancement of tissue-specific fucntion at post-transcriptional levels (Jefferson et al., 1984).

A second possibility is that the shape of the hepatocyte and its internal organization may exist in a critical state in the mature tissue, and this somehow influences or maintains specific gene transcription in the cell nucleus. A relationship between tissue-specific mRNA production and cell shape has been indicated for cultured adipocytes, for example (Spiegelman and Ginty, 1983). If the complex internal organization of the hepatocyte is linked to the geometry of extracellular contacts, simply exposing the cells to extracellular constituents in an abnormal geometry might not immediately result in a resumption of mature tissue-specific function.

However they are generated, how might signals for maximum 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 synthesized 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 of tissue-specific genes, or with other constituents of chromatin, to increase the activity of RNA polymerases on these genes. For example, intracellular phosphorylation of specific factors might serve a regulatory role (Murdoch et al. 1982). No soluble protein that regulates tissue-specific transcription, let alone any phosphorylated structural element that plays a transcriptional role, has yet been isolated, however.

The alternative, especially if maximum transcription depends on intracellular organization or cell shape, is that the molecular mechanism of transcriptional amplification might operate directly through nuclear structure or geometry. Perhaps specific genes interact with a structure that provides increased access to RNA polymerase II (e.g., Jost and Seldran, 1984), and that is disrupted when the cell is removed from the intact tissue. For example, the spatial arrangement of Drosophila melanogaster chromosomes in salivary glands has been traced in serial sections of a number of cells (Mathog et al., 1984), and the arrangement

and nuclear attachment sites appear to be very similar within cells of a common type.

Of course, both soluble factors and structural elements might participate in the regulation of liver-specific transcription. A simple hypothesis derived from this work that integrates both is that a diffusable factor (the "activator" suggested above) binds to regulatory sequences in the DNA of liver-specific genes to result in their initial activation (or to protect them from subsequent repression mechanisms). Genes marked by this factor are then able to interact (perhaps through the factor, e.g., a separate domain) with a nuclear structure that results in increased access to RNA polymerase II, and that is dependent on other aspects other the mature cell/tissue structure.

Science proceeds on the wheels of technology, and the technical challenge of studying "solid-state" characteristics of a cell are daunting. Thus the search for soluble factors that enable or enhance the transcription of groups of genes is surely the next frontier in our understanding of the coordinate control of tissue-specific genes.

CONCLUSION

Work described in this thesis has demonstrated three fundamental points. First, that genes related only in their tissue-specific pattern of expression respond in a concerted fashion to either physical or genetic manipulations of liver cells. Second, that maximum, concerted transcription of liver-specific genes depends on maintenance of the cells in a mature, intact tissue; this implies that a cellular mechanism exists to transmit a signal generated through the tissue structure itself to the transcriptional apparatus in the cell nucleus. Third, that hepatoma cells outside the tissue organization can continue to produce low levels of liver-specific mRNA, distinguishable from complete absence of the mRNAs in other related cell lines; both transcriptional and post-transcriptional mechanisms may be involved in the regulation of this low output of tissue-specific mRNA.

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