Cell–cell and cell–matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes

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ABSTRACT Freshly isolated adult rat hepatocytes exhibit a flat, extended morphology when cultured on dried rat tail collagen in the absence of growth factors; they actively synthesize DNA and express high levels of cytoskeletal mRNAs and proteins (actin, tubulin, cytokeratins, vinculin, α-actinin, and desmoplakin), while exhibiting low levels of liver-specific mRNAs (albumin, α,-inhibitor III, and α,-antitrypsin) and limited synthesis and secretion of albumin. Hepatocytes cultured on hydrated gel matrix from the Engelbreth-Holm-Swarm (EHS) mouse tumor form small spherical aggregates and exhibit low DNA, cytoskeletal mRNA, and protein synthesis, while at the same time exhibiting elevated liver-specific mRNAs and albumin production; these cells, therefore, more nearly conform to the program of gene expression seen within the normal animal. Hepatocytes on hydrated rat tail collagen resemble those on dry collagen when cultured at low density, but at high density they form compact trabecular aggregates, synthesize negligible amounts of DNA, and maintain a pattern of gene expression resembling that of hepatocytes seeded on the EHS matrix. If cell morphology is compact, as on EHS or on hydrated rat tail collagen when densely populated, DNA synthesis and expression of cytoskeletal genes are low, while liver-specific mRNAs are abundant. When cells are extended the opposite is the case. Without the growth supplement DNA synthesis is low throughout but gene expression is little affected. These studies point to the importance of cell–cell and cell–matrix interactions in determining the differentiated phenotype of hepatocytes, and they reveal an inverse relationship between cytoskeletal and liver-specific protein expression.

Studies in a variety of cell culture systems suggest that changes in cell morphology are involved in the regulation of growth and gene expression and in the maintenance of differentiated functions (for reviews, see refs. 1 and 2). Cell morphology reflects the organization of the intracellular cytoskeletal network, which is partially determined by the interaction between the cells and their surrounding extracellular matrix (ECM) (for reviews, see refs. 3–5). This suggests that the ECM influences the expression of differentiated functions through organization of the cytoskeleton (6, 7). Several studies in vitro have demonstrated cytoskeletal involvement in transmitting extracellular signals (mitogenic and differentiation-specific) that regulate various programs of gene expression (for reviews see refs. 1 and 2). It is not known as yet whether mechanisms that regulate the cytoskeleton, both at the level of gene expression and synthesis and at the level of organization of its proteins, are important components of the differentiation programs of highly specialized cells.

It has been demonstrated that the expression of certain differentiated functions in cultured hepatocytes is dependent on the interaction of these cells with constituents of the ECM (8–13). Evidence suggests that particular cytoskeletal genes may undergo extensive changes in expression along with alterations in hepatocyte differentiation (14–16). We have therefore exploited the hepatocyte culture system to study the influence of the ECM on the expression of a defined set of genes. In this study we show that the culture of hepatocytes on a reconstituted gel ECM from the Engelbreth-Holm-Swarm (EHS) mouse tumor (17) differentially regulates the expression of the major cytoskeletal genes (those for actin, tubulin, and cytokeratins) and of several liver-specific genes. DNA synthesis, which is also strongly influenced by the ECM and by cell density, is not closely coordinated with this differential regulation of gene expression.

METHODS

Cell Culture. Hepatocytes were isolated by perfusion of rat livers with collagenase (18), in buffers (19) that were modified to contain 1 mM calcium when collagenase was added. They were cultured in Williams E medium, modified by substitution of ornithine for arginine, and addition of dexamethasone (5 nM), gentamycin (30 µg/ml), ascorbic acid (50 µg/ml), fresh daily), and a growth supplement consisting of insulin (20 milliunits/ml), epidermal growth factor (10 ng/ml), and sodium pyruvate (20 mM). Hepatocytes were plated at 10² (sparse) or 10³ (dense) cells in 1.5 ml of culture medium into 35-mm Lux plastic dishes coated with (i) rat tail tendon dissolved in acetic acid and allowed to dry (crude type I collagen, 0.6 mg per dish) (18), (ii) the same material in the form of a gel (prepared as described in ref. 20), or (iii) a gel prepared from the EHS tumor (17), which is composed primarily of laminin, type IV collagen, and heparan sulfate proteoglycan (1.2 mg of protein per dish). The medium was changed after 1 hr and at 24-hr intervals thereafter.

DNA synthesis was determined after 24-hr incorporation of [³H]thymidine [1 µCi/ml, 360 µCi/mmol; 2.75 µM thymidine (1 Ci = 37 GBq)] (18).

Cell Labeling, Fractionation, and Polyacrylamide Gel Electrophoresis. At selected times after plating, cells were labeled for 2 hr with [³⁵S]methionine (100 µCi/ml, 1121 µCi/mmol) in otherwise methionine-free medium, to which the growth supplement was added. The medium was collected and the secreted proteins were recovered by precipitation with ethanol (2 vol). The cells were then removed from the various matrices by incubation at 37°C in Dulbecco's phosphate-buffered saline (PBS) containing trypsin (0.02%), EDTA (5 mM), and collagenase (2 mg/ml), washed in PBS, and lysed in a buffer containing Triton X-100 (0.5%).

Abbreviations: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; α13, α1-inhibitor III.
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KCl (0.6 M), 2-mercaptoethanol (14 mM), EGTA (2.5 mM), phenylmethylsulfonyl fluoride, and Hepes, pH 7.4 (10 mM), and mixed vigorously. Centrifugation at 15,000 \times g for 2 min yielded a Triton X-100/high-salt-soluble fraction and an insoluble fraction, enriched in intermediate filaments.

Samples were analyzed by sodium dodecyl sulfate (Na-DodSO4)/gel electrophoresis (21) or by two-dimensional gel electrophoresis as described by O'Farrell (22).

The identification of the actin-associated proteins, vinculin and \( \alpha \)-actinin, and the desmosomal plaque protein, desmoplakin, was obtained by means of immunoblotting, where specific proteins were separated on two-dimensional gels and detected with monoclonal antibodies as demonstrated previously (7, 23).

**RNA Extraction, Blot Transfer, and Filter Hybridization.**

Isolation and blot hybridization analysis of total RNA were performed as described elsewhere (24). The various RNA blots were probed with the following: actin, 1.5-kilobase (kb) \( \beta \)-actin cDNA (25); RBT3, 1.7-kb \( \beta \)-tubulin cDNA (26); histone 3.2, \( \alpha \)-lactalbumin; \( \alpha \)-actinin, 2.5-kb cDNA clone (24); albumin, 2.0-kb cDNA (a gift from Douglas Cooper, Whitehead Institute, Cambridge, MA); cytokeratin 18, 1.5-kb cDNA (28); \( \alpha \)-antitrypsin, 1.35-kb cDNA (a gift from Harvey Lodish, Whitehead Institute).

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**RESULTS**

**Culture of Hepatocytes on Dried Rat Tail Collagen Enhances the Synthesis of Cytoskeletal Proteins but Reduces the Synthesis of Albumin.** Freshly isolated hepatocytes were initially cultured in dishes coated with dried rat tail collagen and analyzed after 24–48 hr. Synthesis of the major cytoskeletal proteins, including actin, tubulin, cytokeratins 8 and 18, vinculin, \( \alpha \)-actinin, and desmoplakin, was markedly increased, as was replication of DNA (Fig. 1). In contrast, the level of newly synthesized albumin, a liver-specific protein, declined significantly between the early (Fig. 1 \( IA \)) and late (Fig. 1 \( ID \)) time periods.

Analysis by immunofluorescence microscopy of the cytoskeletal elements in hepatocytes cultured for 48 hr revealed that actin was organized into a cortical ring at areas of cell–cell contact as well as into elaborate stress fibers at the ventral aspects of the cells. These cells also contained an extensive microtubular network. Desmoplakin and cytokeratins were evident at points of cell–cell contact (not shown), whereas vimentin was not detected.

We conclude that the culture of hepatocytes on dried rat tail collagen in growth factor-supplemented medium results in a coordinate increase in the synthesis of the major cytoskeletal elements, accompanied by their organization into elaborate cytoplasmic networks.

**Hepatocytes Cultured on EHS Matrix Maintain a Low Level of Cytoskeletal Protein Synthesis but High Levels of Hepatic Protein Synthesis and Secretion.** An extracellular matrix derived from the EHS tumor (17) has been successfully used to preserve high levels of albumin secretion in cultured hepatocytes (12). We found that whereas hepatocytes cultured on dried rat tail collagen acquired a flat and extended morphology (Fig. 2A), on the EHS matrix they remained round and formed small aggregates that adhered strongly to the matrix (Fig. 2C). Kinetic studies showed that, with increasing times in culture on rat tail collagen, there was a gradual increase in the synthesis of actin and cytokeratins (Fig. 3 \( I \), lanes A, C, and E), while between 24 and 48 hr in culture hepatocytes on EHS matrix synthesized these cytoskeletal proteins at a low rate (Fig. 3 \( I \), lanes B, D, and F).

Two-dimensional gel analysis of \( [35S] \)methionine pulse-labeled proteins revealed that, in addition to the synthesis of cytokeratins (Fig. 3 \( I \), gels \( E' \) and \( F' \)) in cells cultured on EHS matrix, the synthesis of actin and tubulin was also significantly lower (Fig. 3 \( 2 \)). Furthermore, compared with cells cultured on rat tail collagen, hepatocytes cultured on EHS matrix synthesized (Fig. 3 \( 2 \)) and secreted (Fig. 3 \( 3 \)) substantially increased amounts of albumin.

**Hepatocytes Cultured on EHS Matrix Maintain Low Levels of Cytoskeletal mRNAs but High Levels of Liver-Specific mRNAs, Similar to the Levels of These mRNAs in the Liver.** The results of blot hybridization analysis (Fig. 4) clearly demonstrate that, in sparse cultures of hepatocytes cultured on rat tail collagen, there was a dramatic increase in the content of mRNAs coding for actin, tubulin, and cytokeratin.

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**Fig. 1.** Increase in DNA and cytoskeletal protein synthesis, but decrease in albumin synthesis, in hepatocytes plated on rat tail collagen. Sparsely seeded hepatocytes (10\(^6\) cells per 35-mm dish) were labeled for 2 hr with \( [35S] \)methionine after 2 hr (\( IA, 2A \)), 8 hr (\( IB \), 24 hr (\( IC, 2C \)), or 48 hr (\( ID, 2D \)) in culture. The Triton X-100/high-salt-soluble fraction (\( I \)) and -insoluble fraction (\( 2 \)), which is enriched in intermediate filaments, were analyzed by the methods of O'Farrell (22) and Laemmli (21), respectively. The amount of DNA synthesis after various times in culture was measured by \( [3H] \)thymidine incorporation (3). IEF, isoelectric focusing; alb, albumin; aa, \( \alpha \)-actinin; v, vinculin; t, tubulin; d, desmoplakin, CK 8 and CK 18, cytokeratins as described by Franke et al. (29); M, molecular mass markers (kDa).
18 (Fig. 4, lanes 2). There was also a high rate of DNA synthesis (Fig. 5), along with histone mRNA accumulation, while expression of the liver-specific mRNAs for \( \alpha_1 \)I3, \( \alpha_1 \)-antitrypsin, and albumin decreased significantly (Fig. 4, lanes 2). In contrast, the RNA from normal adult liver (Fig. 4, lanes 1) and from cells cultured on EHS matrix (Fig. 4, lanes 3) contained low levels of cytoskeletal protein mRNAs and histone mRNA but high levels of the liver-specific mRNAs.

**Population Density of Hepatocytes Plated on Hydrated Collagen Gels Influences the Synthesis of Cytoskeletal and Liver-Specific Proteins.** To define the nature of the signal responsible for the maintenance of a more differentiated phenotype on particular ECMs, hepatocytes were cultured on uncoated dishes and on dishes coated with various ECM components (laminin, fibronectin, collagen types I or IV), and also with poly(L-lysine). On all of these substrata hepatocytes had an extended flat morphology and synthesized actin, tubulin, cytokeratins, and liver-specific proteins at levels similar to those found in cells plated on dried rat tail collagen (data not shown).

Since the EHS matrix was presented to the cells in the form of a hydrated gel, hepatocytes were also cultured for 48 hr, at high and low densities, on hydrated rat tail collagen gels. Unlike hepatocytes cultured on dry rat tail collagen (Fig. 2D), hepatocytes seeded at high density on hydrated collagen gels displayed a compact trabecular organization (Fig. 2E); these cells expressed a pattern of cytoskeletal protein synthesis (Fig. 6d and e-dense, lane 2) and albumin synthesis (Fig. 6d) and secretion (Fig. 6 f-dense, lane 2) similar to those of cells cultured on the EHS matrix (Fig. 6 b and e- and f-dense, lanes 3). In contrast, hepatocytes cultured at low density on hydrated collagen gels had an extended morphology (Fig. 2B) and exhibited high levels of cytoskeletal protein synthesis (Fig. 6 c and e-sparse, lane 2).

**DISCUSSION**

Studies by others have indicated that maintenance of the differentiated phenotype of hepatocytes in cell culture is dependent upon a combination of particular ECM components and hormonally defined media (12, 13). The results presented here are consonant with these findings, but they
we show that cell-cell gene expression. Taken together, these results strongly support the idea that the organization of the cytoskeleton, which is dictated by the extent of cell–cell and cell–matrix interaction, is intimately associated with mechanisms that regulate tissue-specific gene expression.

The use of the different cell culture systems has revealed a significant degree of plasticity within the program of mechanisms that regulate hepatocyte gene expression. It appears that we can control the same pattern of gene expression either (i) by manipulating the extent of cell–cell interaction by plating hepatocytes at different densities on hydrated collagen gels or (ii) by culturing cells at any density on a reconstituted basement membrane extracted from the EHS tumor. One common parameter that both systems share is a dramatic change in cell shape, implying that cell shape may be a primary regulator of phenotypic expression. Similar findings have been reported in a variety of other experimental systems (1, 2, 5). Since the EHS matrix is capable of preserving the differentiated phenotype in hepatocytes at all culture densities, it is possible that differentiation is the result of the interaction of cells with specific components of the ECM [e.g., glycosaminoglycans and heparan sulfate proteoglycans, as suggested by Spray et al. (32)], thereby inducing the required morphological change. It is also possible that cell shape is simply a manifestation of the culture conditions and plays no direct role in regulating gene expression. Instead, the predominant mode of regulation may depend upon mechanisms that are intimately associated with the extent of cell–cell and/or cell–matrix interaction. There is now accumulating evidence that these interactions are dependent upon specific transmembrane receptors that link extracellular and intracellular elements (e.g., integrin connects fibronectin to the microfilament system for review, see ref. 33). Signals may therefore be transmitted from these various receptors through a common set of cytoskeletal elements and converge on similar regulatory systems.

The results presented here provide a dramatic example of the coordinate regulation of the expression of all major cytoskeletal proteins during differentiation. Whether such regulation is directly involved in mechanisms that determine the differentiated phenotype of hepatocytes is presently difficult to assess. It is possible that the dramatic changes in
cytoskeletal gene expression may initiate the reorganization of the cytoskeleton and in doing so affect the expression of liver-specific genes, as suggested in other differentiation systems (34). Alternatively, this regulation may be totally unrelated to the differentiation program and may result from the alteration of cell morphology. These alterations could in turn induce a change in the organization of the cytoskeleton, triggering feedback or autoregulation mechanisms that control cytoskeletal gene expression (35-41).

During the short culture periods analyzed in this study, hepatocytes cultured either on hydrated collagen gels at high density or on the EHS matrix appeared to express the same general pattern of gene regulation. It is possible, however, that these two cell culture systems do regulate gene expression by totally different means (i.e., transcriptional vs. posttranscriptional) and that the cell culture period was not long enough to detect these differences. The EHS matrix is known to preserve the differentiated phenotype over longer periods of culture (12), and therefore, it is necessary to extend our culture period to study the later stages of the dedifferentiation program.

Recent studies have revealed that there are multiple mechanisms that regulate the various liver-specific proteins, and these mechanisms differ from protein to protein. For instance, while the decrease in albumin expression on dry rat tail collagen is transcriptional, significant levels of albumin mRNA are maintained by RNA stabilization (14, 24). This is not the case, however, for the expression of α1I3. The decrease in transcription of this gene, resulting from the dedifferentiation process, is accompanied by a corresponding rapid decrease in mRNA in the cytoplasm (24). Expression of α1I3 appears to be hormonally controlled. It is likely, therefore, that complete maintenance of the liver-specific phenotype of hepatocytes will require many interacting systems, including both extracellular matrices and a plethora of hormones and factors. Nevertheless, a continued study of the effect of extracellular components on the expression of a defined set of genes will contribute significantly to our basic understanding of the problem. For instance, it will be important to determine whether, in hepatocytes cultured on the EHS matrix for longer periods of time, the transcription of albumin and other liver-specific proteins is restored to the levels found in the liver and whether proteoglycans and glycosaminoglycans are the ECM components responsible for the restoration of such regulation, as suggested by Fujita et al. (42).

What role can the cytoskeleton play in such regulation? It may be involved in the posttranscriptional regulation of mRNA half-lives by modulating the interaction of the polyribosomes with various elements of the cytoskeleton, as suggested by previous studies (for review, see refs. 3 and 43).

The molecular mechanisms by which changes in cell–cell and cell–matrix contacts affect the organization and expression of the cytoskeleton, in such a way as to maintain the differentiated phenotype, will have to be determined. The identification and characterization of transmembrane receptors that link extracellular and cytoskeletal elements will provide us with additional tools to address questions regarding the initial steps in this process at the molecular level.

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