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The Extracellular Matrix Coordinately Modulates Liver Transcription Factors and Hepatocyte Morphology

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The extracellular matrix (ECM) promotes tissue morphogenesis, cell migration, and the differentiation of a variety of cell types. However, the mechanisms by which ECM causes differentiated gene expression have been unknown. In this report, we show that culturing the hepatocyte-derived cell line H2.35 on an ECM gel changes cell morphology and selectively stimulates the transcription of a subset of liver-specific genes, including serum albumin. Transcriptional activation by ECM also occurs with transfected plasmids bearing the transcriptional enhancer of the albumin gene. ECM substrates of different composition activated the albumin enhancer only when the ECM promoted a cuboidal, differentiated cell morphology. Enhancer activation by the ECM was mediated by two liver transcription factors, HNF3α and eH-TF, which appear to be regulated differently by matrix. Specifically, we found that a collagen gel substratum caused a selective increase in the factor HNF3α at the levels of mRNA accumulation and DNA-binding activity in nuclear extracts, both in H2.35 cells and in the hepatoma cell line HepG2. We conclude that the ECM can stimulate cell differentiation by selectively activating transcriptional regulatory factors and that such regulation occurs coordinately with ECM-promoted changes in cell shape.

Cell adherence to an extracellular matrix (ECM) is vitally important for proper tissue development, and a direct role has been proposed for the ECM in regulating gene expression and maintaining cell differentiation (reviewed in references 2 and 32). The molecular components of the ECM, including collagens, glycoproteins, and proteoglycans, are physically interconnected with proteins of the cytoskeleton via transmembrane receptors such as integrins (18, 19, 42); these mechanical interactions appear important in determining cell shape (20, 48). ECMs reconstituted in vitro have been shown to coordinately maintain the differentiated cell morphology and function of many cell types, including mammary epithelial cells (29, 43), retinal epithelial cells (35), pancreatic acinar cells (1), neural cells (7), and bone cells (47), suggesting that matrix-dependent changes in cell shape have a general role in determining or maintaining tissue differentiation. Protein factors associated with the ECM, such as epidermal growth factor and transforming growth factor α, have also been implicated in matrix-induced cell differentiation (40, 45). Thus, the ECM may affect differentiation via distinct pathways. However, it is unknown how cellular interactions with the ECM lead to the selective expression of differentiated functions.

Primary hepatocyte cultures have been a useful system for studying the effects of ECM on cell differentiation. When hepatocytes are disaggregated from the liver and cultured in vitro on resilient ECM-derived gels containing either type I collagen or basement membrane proteins, the substratum permits the cells to assume their differentiated shape and they express high levels of mRNA for liver-specific genes, including serum albumin (3, 41, 44). By contrast, when hepatocytes are cultured on plastic surfaces, they exhibit a dedifferentiated, flattened shape, due to the inelasticity of the substratum (44), and liver-specific gene transcription declines dramatically (10). The proximity of the aforementioned ECM components to hepatocytes in the liver (4, 31) suggests that they play a relevant role in vivo.

While the studies mentioned above discovered that ECM can regulate albumin mRNA stability, subsequent studies demonstrated that culturing hepatocytes on an ECM can stimulate albumin gene transcription (5, 8). We previously showed that liver-specific gene expression was increased in a conditionally transformed, hepatocyte-derived cell line, H2.35, by a complex culture environment that included an ECM-derived gel matrix (52). Specifically, we demonstrated a significant increase in albumin gene transcription, and a much larger increase in albumin mRNA stability, when H2.35 cells were cultured at the restrictive temperature for simian virus 40 (SV40) large T antigen, in a hormonally defined, serum-free medium, and on a type I collagen gel (differentiating conditions). The transcriptional effects were mediated, in part, by an upstream enhancer element (52). We also showed that the binding of the liver-enriched transcription factors, eE-TF, eH-TF (53), and a member (or members) of the HNF3 family of proteins (12, 27) was essential for activating the albumin enhancer in differentiated H2.35 cells (30). The DNA-binding activities of these factors were low in nuclear extracts from H2.35 cells cultured on plastic, at 33°C, and in the presence of serum but were increased significantly by culturing the cells under differentiating conditions (30). Interestingly, only HNF3 DNA binding activity was increased by culturing the cells on a collagen gel matrix alone, indicating that the transcription factors are regulated by distinct signals. The HNF3 proteins have recently been shown to be members of a regulatory family that includes the product of the *Drosophila* homeotic gene fork head (27, 49, 50).

To understand the cell-ECM interactions important for transcriptional activation, in this report we assess the ability of different ECM substrata to independently activate the albumin enhancer. We also find that the ECM modulates HNF3 factors in different hepatic cell lines and that an additional transcription factor mediates the enhancer's response to ECM. Finally, we examine whether enhancer

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activation is linked to changes in cell shape promoted by the ECM. Our results implicate distinct transcriptional mechanisms in the control of cell differentiation by ECM. These studies are the first to define specific transcription factors that mediate the influence of ECM on gene expression.

MATERIALS AND METHODS

Transcriptional run-on and Northern (RNA) blot analyses. Transcriptional run-on assays were performed as described previously (52). [^{32}P]RNAs (2×10^6 cpm) were hybridized to a genomic clone of the albumin gene, to cDNA clones of liv-S1 (13) and tubulin (11), or to plasmid pBR322 as a non-specific control. [^{32}P]RNA (8×10^4 cpm) was also hybridized separately to DNA encoding rRNA. Specific hybrids were detected by autoradiography and quantitated by scanning different exposures with an LKB laser densitometer. Northern blots were prepared as described previously (52), using either total RNA or poly(A)$^+$ RNA selected by chromatography on oligo(dT)-cellulose; RNA was hybridized to a nick-translated cDNA probe of albumin (23) or HNF3α (26). The HNF3α probe was from the 5′ *Eco*RI site to a *Hin*cII site just downstream of the DNA-binding domain; this probe cross-hybridizes to HNF3β and -γ transcripts (27). After autoradiography, the RNAs were rehybridized to a cDNA probe for β-actin (11) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 46). mRNA levels were quantitated by laser densitometry scanning of different autoradiographic exposures.

Plasmid constructions. Plasmids pAT2 and pRT1 have been described previously (52) and contain the mouse albumin promoter (positions −787 to +8 with respect to the transcription start site) and the Rous sarcoma virus long terminal repeat, respectively, fused to a thymidine kinase (*tk*) reporter gene. An 830-bp *Nhe*I-to-*Ava*II restriction fragment from the albumin enhancer (38) was filled in and ligated into pAT2 at a filled-in *Eco*RI site adjacent to the albumin promoter position −787, to create pAT2-NA. The SV40 enhancer was ligated into the *Eco*RI site of pAT2, to create pAT2-SV. Substitution mutations of the binding site for eE-TF, HNF3, or eH-TF were generated within the 830-bp albumin enhancer fragment as described elsewhere (30, 53). To generate binding site multimers, we tandemly ligated double-stranded oligonucleotides with complementary 5′ extensions of 3 bp and which encompassed the binding sequence for HNF3 or eH-TF from the albumin enhancer (30); the sequences for the oligonucleotides are provided below. The resulting multimers were then filled in and ligated into pAT2 at the filled-in *Eco*RI site. The number and integrity of binding sites in each plasmid were confirmed by dideoxy sequencing.

Preparation of extracellular substrata and cell culture. Rat tail type I collagen gels (Collaborative Research, Waltham, Mass.) were prepared as described previously (52); nongelled collagen plates were prepared by coating 60-mm dishes with 100 μg of type I collagen before cells were seeded. Similar results were obtained with commercially coated collagen plates (Collaborative Research; data not shown). Gels derived from the Engelbreth-Holm-Swarm (EHS) mouse tumor (36) (EHS gels) were prepared by adding 1 ml of basement membrane Matrigel (Collaborative Research) per 60-mm dish; gelation was at 37°C for 1 h. To reduce the levels of soluble contaminants, all ECM gels were conditioned with medium which was changed after 20 h, prior to seeding of cells. H2.35 cells were cultured at 33°C at subconfluent densities in basal medium (52), which consisted of Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 4% fetal bovine serum (FBS; HyClone, Logan, Utah) and 0.2 μM dexamethasone, or at 39°C in serum-free medium prepared as described previously (15) and supplemented with 0.2 μM dexamethasone. HeLa and NIH 3T3 cells were cultured at 37°C in DMEM containing 6% calf serum and 4% FBS. HepG2 cells were cultured at 37°C in DMEM and Ham F12 nutrient mixture (GIBCO) at a ratio of 1:1, with 10% FBS and 10 μg of bovine pancreatic insulin (Sigma, St. Louis, Mo.) per ml. All media contained penicillin and streptomycin, each at 100 U/ml.

Transient transfection and primer extension analysis. Cells were transfected with pAT2 derivatives and the internal control plasmid pRT1 as described previously (52). Total cellular RNA was isolated, and specific RNA levels from test and control plasmids were quantitated simultaneously with a primer extension assay using a labeled oligonucleotide complementary to the common *tk* reporter transcript (52). For the subculture experiments shown in Fig. 2d, H2.35 cells were transfected on plastic for 24 h and then subcultured onto plastic dishes, collagen gels, or EHS gels for an additional 48 h before isolation of total RNA. H2.35 cells were also transfected on plastic or on nongel, type I collagen substrata for 24 h; then the medium was aspirated, and the cells were rinsed twice with DMEM. Next, the cells were fed with 3.5 ml of basal medium (per 60-mm dish) supplemented with EHS proteins at 500 μg/ml. A thin gel formed over the cells during a 24-h period (8), after which the cells were refed basal medium lacking EHS proteins. RNA was harvested from the cells after an additional 24-h incubation.

Preparation of nuclear extracts; in vitro translation. H2.35 cells, HepG2 cells, and HeLa cells were seeded onto 150-mm plastic plates or type I collagen gels at the same densities as used in the transfection assays and cultured as described above for 3 days, with a daily change of medium. Cells cultured on 150-mm plastic plates were rinsed with PBS (phosphate-buffered saline) and then removed from plates with a 0.1% trypsin solution at room temperature. Cells cultured on 150-mm type I collagen gels were rinsed with PBS, treated in situ with 1,000 U of collagenase (type VII; Sigma) per plate in 2 ml of DMEM at 37°C for 5 min, and collected by centrifugation. Cells were washed and nuclear extracts were isolated by a procedure adapted from Dignam et al. (14) as described elsewhere (30). HNF3α protein was produced by translating a T7 polymerase transcript from the HNF3α cDNA clone (26), using a nuclease-treated rabbit reticulocyte lysate (Promega Biotec).

Electromobility shift assays. Binding reactions were performed as described previously (30) except that the reaction mixtures contained 3.0 to 7.5 μg of nuclear extract in a 15-μl solution of 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% Ficoll, with 750 ng of poly (dI-dC) (per 7.5 μg of extract) and a 100-fold molar excess of competitor oligonucleotide (as indicated). The mixtures were incubated at room temperature for 20 min, 0.1 ng (5×10^4 to 10×10^4 cpm) of oligonucleotide probe was added, and incubation was continued for an additional 20 min prior to electrophoresis. Binding to the eH oligonucleotide differed in that (i) the reaction mixture contained 900 ng of poly(dI-dC) and 60 mM NaCl and (ii) electrophoresis was performed in a solution of 200 mM glycine, 25 mM Tris base, and 1 mM EDTA. Oligonucleotide probes were labeled with ^{32}P-deoxynucleoside triphosphates by filling in the ends with Klenow polymerase I. Double-stranded oligonucleotides used as probes or competitors were composed of the follow-

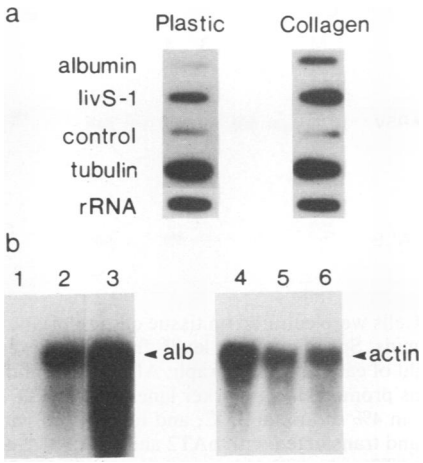

FIG. 1. Stimulation of albumin gene transcription by culturing H2.35 cells on a collagen gel. (a) Transcriptional run-on analysis of H2.35 cells cultured on conventional tissue culture plastic or on a gel substratum of type I collagen. Both conditions were at 33°C and in serum. (b) Steady-state levels of albumin (alb; lanes 1 to 3) and actin (lanes 4 to 6) mRNAs, revealed by Northern blot analysis of RNAs isolated from H2.35 cells cultured on substrata of plastic (lanes 1 and 4) or a collagen gel (lanes 2 and 5) at 33°C and in serum, or on a collagen gel at 39°C and in a hormonally defined, serum-free medium (15) (lanes 3 and 6).

ing mouse albumin sequences (top strands shown), flanked at each end by three extra base pairs (underlined): eG/HNF3 site, GCTCCAGGGAATGTTTGTTCTTAAATACCATCG CT; eH-TF site, GCTCCGAACGTGTTTGCCTTGGCCAG TTTTCCATGTACATGCAGCT; promoter CCAAT site, CT CGGGGTAGGAACCAATGAAATGCGAGGTAAATC; and promoter HNF1 site CAGTGGTTAATGATCTACAGTT ACAG. The oligonucleotide of the HNF3 binding site from the transthyretin (TTR) promoter was from −85 to −111 (12).

RESULTS

Albumin gene transcription is induced by culturing H2.35 cells on a type I collagen gel. We derived the H2.35 cell line from mouse hepatocytes immortalized with a strain of SV40 that expresses a temperature-sensitive large T antigen (9). During routine propagation of H2.35 cells at the permissive temperature (33°C), on a plastic substratum, and in 4% FBS, albumin gene transcription was below our level of detection (Fig. 1a; 52). We previously showed that the transcription rates of albumin and certain other liver genes could be induced by culturing H2.35 cells at the restrictive temperature (39°C) and on a biological matrix consisting of a type I collagen gel while the cells were in hormonally defined, serum-free medium (52). To determine whether culturing H2.35 cells on a type I collagen gel, but at the permissive temperature (33°C) and in serum, was sufficient to stimulate albumin transcription, we performed a nuclear run-on assay and used the resulting [^{32}P]RNAs to probe cloned sequences bound to a filter. As seen in Fig. 1a, culture on a collagen gel for 3 days stimulated the transcription of genes encoding albumin and a liver-specific major urinary protein (liv-S1), whereas the transcription of tubulin and ribosomal DNA was unaffected. The transcription of albumin was increased at least threefold over background in response to the matrix and was accompanied by a dramatic increase in the steady-state level of albumin transcripts (Fig. 1b, lanes 1 and 2). Maximum albumin mRNA levels were observed in H2.35 cells shifted simultaneously to a collagen gel, at 39°C, and in serum-free medium (15) (Fig. 1b, lane 3), showing that the effects of the shift in temperature and medium are at least partly additive to those of collagen. We consistently observed a decrease in actin mRNA in H2.35 cells cultured on collagen (Fig. 1b, lanes 4 to 6); the reciprocal relationship between albumin and actin expression is also seen in primary hepatocyte cultures on ECM gels (3, 41).

The liver-specific albumin enhancer is activated selectively in H2.35 cells by culture on a type I collagen gel. To test the ability of a collagen gel substratum to stimulate albumin regulatory sequences, the reporter plasmids pAT2, which contains the liver-specific albumin promoter (16, 37), and pAT2-NA, a derivative with the albumin enhancer (38) upstream of the promoter, were cotransfected into H2.35 cells with the control plasmid pRT1; pRT1 contains the Rous sarcoma virus promoter. Two days later, total RNA was isolated and transcription of the common *tk* reporter gene from the different transcription start sites was quantitated simultaneously with a primer extension assay. This strategy eliminated any selective effects on mRNA stability. As seen previously (52), the presence of the albumin enhancer did not stimulate the promoter in H2.35 cells cultured on a plastic substratum, at 33°C, and in serum (Fig. 2a, lanes 2 and 3). However, when normalized to the Rous sarcoma virus promoter control, the enhancer reproducibly stimulated the albumin promoter fourfold in H2.35 cells cultured on a collagen gel matrix, at 33°C, and in serum (Fig. 2a, lanes 4 and 5). The enhancer was also activated by collagen when placed downstream of the reporter gene (data not shown), proving that the enhancer sequence was the responsive element. We also found that the enhancer was activated by culturing H2.35 cells on a plastic substratum, but at 39°C and in serum-free medium (Fig. 2a, lanes 6 and 7), demonstrating that stimuli other than ECM can activate the enhancer.

Next, we examined whether culturing H2.35 cells on a collagen gel could stimulate a different enhancer that functions in a variety of cell types. This experiment also addressed whether the ECM affected the ability of the albumin promoter to respond to enhancer elements in general. The SV40 enhancer readily stimulated the albumin promoter in H2.35 cells cultured on a plastic substratum (Fig. 2b, lanes 1 and 2); however, the activity of the SV40 enhancer was not increased by culture on collagen (Fig. 2b, lanes 3 and 4) and in some experiments appeared weaker. We conclude that the stimulatory effect of a collagen gel is selective for the albumin enhancer and that it does not affect the promoter's response to enhancers in general.

To address whether activation of the enhancer by ECM was cell specific, we transfected nonliver cell lines cultured on collagen gels. The enhancer was inactive when transfected into human epithelial HeLa cells (Fig. 2c, lanes 1 and 2), mouse fibroblastic NIH 3T3 cells (Fig. 2c, lanes 3 and 4), and mouse fibroblastic L cells (data not shown). Thus, regulation of the albumin enhancer by collagen was restricted to cells of the hepatocyte lineage; the basis for the specificity is addressed below (see Fig. 5 and 6). The basal expression of the albumin promoter in nonliver cell lines has been described previously (52) and is analogous to the expression of other cell-specific promoters when transfected into heterologous cell lines. We also transfected the human hepatoma cell line HepG2, which synthesizes a number of liver-specific proteins, including albumin (25). However, the albumin enhancer was inactive in HepG2 cells cultured on a

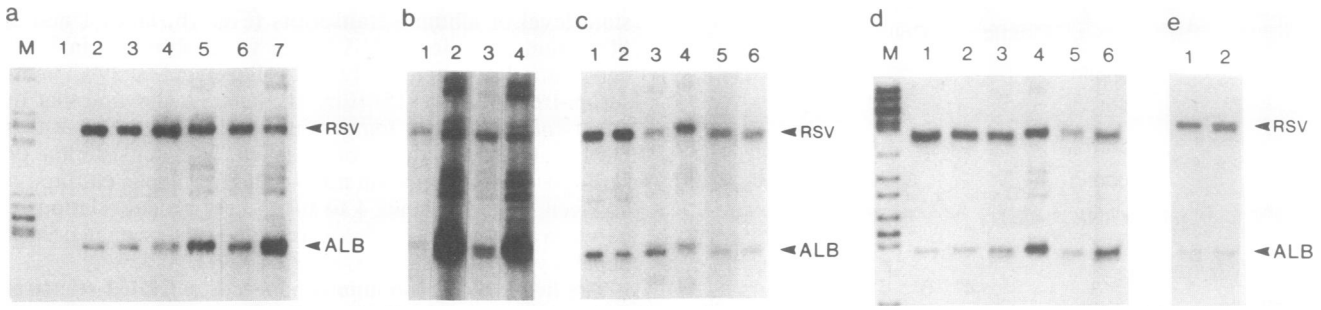

FIG. 2. Selective activation of the albumin enhancer in H2.35 cells by ECM gels. Cells were cultured on tissue culture plastic or on ECM-derived substrata and transfected with albumin test plasmids and internal control plasmids. Specific mRNA levels from *tk* reporter genes were quantitated with a primer extension assay. Extension products are indicated at the right of each autoradiograph: ALB (pAT2 and derivatives, containing the albumin promoter) and RSV (pRT1, containing the Rous sarcoma virus promoter). M, marker lanes showing DNA sequence ladders. (a) Lanes: 1, mock-transfected H2.35 cells; 2 and 3, H2.35 cells on plastic, in 4% serum, at 33°C, and transfected with pAT2 and pAT2-NA, respectively; 4 and 5, H2.35 cells on collagen gels, in 4% serum, at 33°C, and transfected with pAT2 and pAT2-NA, respectively; 6 and 7, H2.35 cells on plastic, in serum-free medium, at 39°C, and transfected with pAT2 and pAT2-NA, respectively. (b) H2.35 cells in 4% serum, at 33°C, on plastic (lanes 1 and 2) or on collagen gels (lanes 3 and 4), and transfected with pAT2 and pAT2-SV, respectively. (c) HeLa cells (lanes 1 and 2), NIH 3T3 cells (lanes 3 and 4), or HepG2 cells (lanes 5 and 6) cultured on collagen gels and transfected with pAT2 and pAT2-NA, respectively. (d) H2.35 cells transfected on a plastic substratum and then subcultured on plastic (lanes 1 and 2), on collagen gels (lanes 3 and 4), or on EHS gels (lanes 5 and 6), with pAT2 and pAT2-NA, respectively. (e) H2.35 cells transfected on nongel collagen-coated dishes with pAT2 (lane 1) or pAT2-NA (lane 2). Each result shown was reproduced two to five times.

collagen gel (Fig. 2c, lanes 5 and 6), demonstrating that not all hepatocyte-derived cell lines retain matrix responsiveness. Other workers have found that the albumin enhancer can function in HepG2 cells on a plastic substratum, but only in the context of an infectious adenovirus vector and while the cells are treated with cytosine arabinoside (17), making it difficult to compare their results with ours. The differences between HepG2 and H2.35 cells in our experimental system are explored in detail below (see Fig. 5 and 6).

Basement membrane ECM components can also activate the albumin enhancer. Hepatocytes in vivo are in contact with basement membrane components such as type IV collagen, laminin, and heparin sulfate proteoglycans, as well as fibronectin, whereas type I collagen provides structural support for interconnecting plates of hepatocytes (4, 31). To determine whether basement membrane proteins could stimulate the albumin enhancer, we cultured H2.35 cells on EHS gels that closely resemble basement membrane ECM in protein constituency (24). We found that H2.35 cells cultured on EHS gels were not transfectable by either the calcium phosphate or lipofection procedure. To circumvent this problem, we transfected test plasmids into H2.35 cells on plastic for 24 h and then subcultured the cells onto EHS gels, collagen gels, and plastic dishes for an additional 48 h. We found that the transfected albumin enhancer was reproducibly activated in H2.35 cells subcultured on EHS gels (Fig. 2d, lanes 5 and 6) and on collagen gels (Fig. 2d, lanes 3 and 4) and was inactive in cells subcultured on plastic (Fig. 2d, lanes 1 and 2). We conclude that different ECM gels can activate the albumin enhancer in H2.35 cells.

Activation of the albumin enhancer occurs when ECMs promote a differentiated hepatocyte morphology. We observed dramatic differences in the morphology of H2.35 cells cultured on the different substrata. Cells grown on tissue culture plastic exhibited a flattened, extended shape (Fig. 3a), while those cultured on type I collagen gels assumed a raised, three-dimensional shape (cuboidal) reminiscent of hepatocyte morphology in vivo, and the cells aggregated into tight clusters (Fig. 3c). By contrast, the flat, extended shapes of HepG2 and HeLa cells did not change significantly when the cells were cultured on collagen gels (data not shown). We also found that H2.35 cells on collagen proliferated at a slower rate than did cells on plastic (data not shown). H2.35 cells cultured on EHS gels exhibited even more compact associations than on collagen gels, and they formed striking, continuous networks (Fig. 3d). The same gel matrices also induced differentiated morphologies and reduced the proliferation rate of primary hepatocyte cultures (3, 5, 34, 44). However, primary hepatocytes cultured on a dried, nongel collagen substratum exhibited a dedifferentiated morphology, and they expressed liver-specific mRNAs at levels much lower than the nearly in vivo levels seen when the cells were cultured on either collagen or EHS gels (3). Thus, tensile properties of the substratum, and not simply protein composition, appear critical in promoting differentiated cell shape and gene expression in hepatocytes.

To test whether cell attachment to ECM components as a rigid substratum could activate the albumin enhancer, we coated plastic dishes with type I collagen (prepared from the same stocks used to make the gels) to form rigid, nongel substrata. When H2.35 cells were transfected on collagen-coated plastic, they exhibited nearly the same flattened, dedifferentiated morphology as did cells on untreated plastic (Fig. 3b), and the albumin enhancer was inactive (Fig. 2e). Identical results were obtained with use of commercially prepared plates coated with collagen in a nongel form and with plates that we coated with EHS proteins in a nongel form (data not shown). We conclude that the malleability of the ECM substratum, rather than solely its chemical composition, is important for ECM to coordinately permit the cuboidal cell shape and activate the albumin enhancer.

It seemed possible that enhancer activation could be caused by soluble factors that remained bound to matrix proteins and whose regulatory activities depended on being presented to the cells in an ECM gel. Indeed, hormones and growth factors are important in certain cases of ECM-induced differentiation (43, 45). To test this possibility, we transfected H2.35 cells either on plastic or on a nongel, type I collagen substratum for 24 h and then supplemented the culture medium with EHS proteins at 0.5 mg/ml, as described by Caron (8). Within several hours, the EHS proteins formed a thin gel on top of the cells. After 48 h of

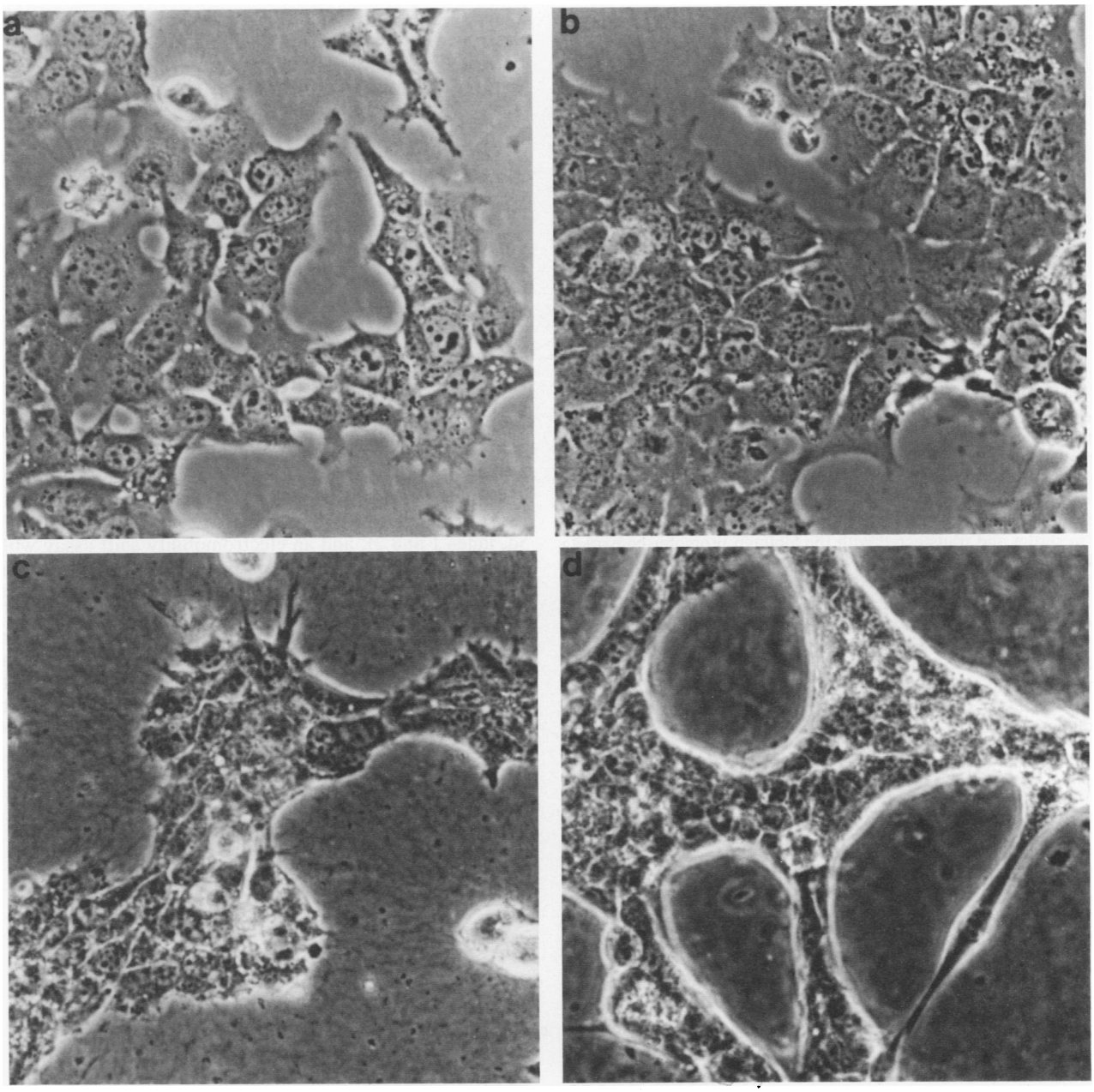

FIG. 3. Morphology of H2.35 cells on different culture substrata. H2.35 cells from the transfection experiments shown in Fig. 2 were photographed prior to RNA isolation. Cells were cultured in 4% serum at 33°C on either plastic (a), plastic dishes coated with nongel type I collagen (b), a type I collagen gel (c), or an EHS gel (d). Magnification at ×400 with phase-contrast optics.

incubation, the H2.35 cells remained flattened and extended, and the transfected enhancer was inactive (data not shown). Thus, exposure to EHS proteins in a gel form, without accompanying changes in cell shape, is insufficient to activate the albumin enhancer in H2.35 cells.

Binding of two liver transcription factors permits enhancer activation by collagen. We previously showed that the binding of HNF3 to the albumin enhancer eG site (Fig. 4a) was severalfold higher in nuclear extracts from H2.35 cells cultured on a collagen gel than in extracts from cells on plastic (30). HNF3 binding activity in liver is now known to consist of three distinct transcription factors, HNF3α (formerly HNF3A), HNF3β, and HNF3γ, that are abundant in liver and in a limited subset of other tissues (26, 27) and that are essential for the transcription of TTR and other hepatic genes (12). Two other factors (Fig. 4) whose binding to the albumin enhancer is increased in fully differentiated H2.35 cells are eE-TF, which binds to the eE site and is similar in binding activity to the liver factor C/EBP, but is a distinct protein (30), and eH-TF, which binds the eH sequence containing the TGTTTGC motif present in the control elements of other liver genes (53). To determine which of these factors activated the albumin enhancer in response to ECM alone, we transfected binding site mutants into H2.35 cells cultured on collagen gels, at 33°C, and in serum. Mutation of the eE site had no effect on enhancer activity (Fig. 4b, lanes

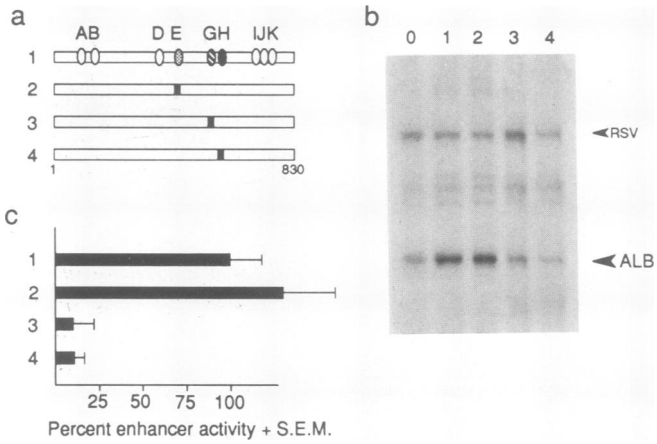

FIG. 4. Evidence that binding sites for HNF3 and eH-TF are necessary for activation of the albumin enhancer by collagen. (a) Constructs used. Open bars represent the 830-bp albumin enhancer segment; ovals on segment 1 indicate binding activities detected in differentiated H2.35 cells (30); black boxes in segments 2 to 4 indicate mutations of sites eE, eG, and eH, respectively. (b) Primer extension of RNAs from H2.35 cells cultured on collagen, at 33°C, and in serum and transfected with the albumin promoter construct pAT2 (lane 0) or with pAT2 derivatives containing the wild-type or mutant enhancer segments fused to the promoter (lanes 1 to 4); lane numbers correspond to numbers for enhancer segments shown in panel a. Primer extension products are indicated as in Fig. 2. (c) Activity of each mutant enhancer as a percentage of that of the wild-type 830-bp enhancer, plus the standard error of the mean (S.E.M.). Average percent activity ± standard error of the mean and number of experiments performed (in parentheses) for each construct were as follows: 1, 100 ± 19 (7); 2, 131 ± 30 (2); 3, 11 ± 11 (3); 4, 11 ± 6 (2).

1 and 2), whereas mutation of either the eG or eH site destroyed activity (Fig. 4b, lanes 3 and 4). We conclude that both HNF3 and eH-TF binding permit the albumin enhancer to respond to collagen, whereas eE-TF, in these studies and others with different constructs (data not shown), appears not to be involved.

The HNF3 and eH-TF binding sites are independently responsive to ECM signals in H2.35 cells. We next examined whether the HNF3 and eH-TF binding sites were sufficient to stimulate transcription in response to collagen. We prepared multimers of enhancer sites eG and eH and fused them to the albumin promoter in pAT2 (Fig. 5a). When H2.35 cells were transfected on a plastic substratum, a single binding site had no effect (data not shown) but two copies of the eG site increased promoter activity an average of fivefold (Fig. 5b, Pl., lanes 1 and 2). Moreover, the eG site dimer stimulated transcription at least 12-fold in H2.35 cells on collagen gels (Fig. 5b, Col., lanes 1 and 2) while the temperature was held constant at 33°C. Thus, culturing H2.35 cells on a collagen gel induces both HNF3 DNA binding and transcriptional activity.

The eG site dimer was unable to enhance transcription in HeLa cells cultured either on plastic or on collagen gels (Fig. 5d, lanes 1 and 2), and an electromobility shift assay with a labeled eG site failed to reveal any HNF3 DNA-binding activity (Fig. 6a, lanes 10 to 12); these findings are consistent with the absence of HNF3 family transcripts in a variety of nonliver tissues (26). By contrast, the eG site dimer stimulated the albumin promoter 30-fold in HepG2 cells transfected on plastic (Fig. 5c, Pl., lanes 1 and 2), similar to the strong effects of multimers of an HNF3 binding site from the TTR gene in these cells (12). No additional stimulation by

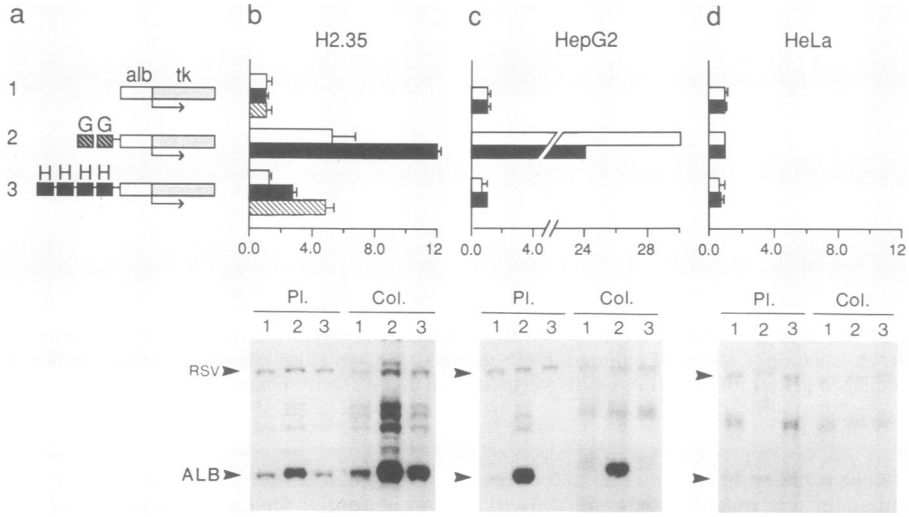

FIG. 5. Induction by collagen of transcriptional activities of HNF3 and eH-TF in H2.35 cells. (a) Schematic representations of the test plasmid pAT2 (construct 1) and derivatives containing multimers of the eG (construct 2) or eH (construct 3) albumin enhancer site fused to the albumin promoter. White box, albumin promoter (alb); stippled box, *tk* reporter gene; striped boxes, eG site (G); black boxes, eH site (H). Arrows indicate transcribed sequences. In panels b to d, the test plasmids shown in panel a were transfected into H2.35 cells (b), HepG2 cells (c), or HeLa cells (d), and reporter transcripts were quantitated by primer extension. The graphs show results from cells cultured on plastic (white bars) or collagen gels (black bars), both at 33°C and in serum, or under full differentiating conditions for H2.35 cells only (striped bars); each error bar indicates the standard error of the mean for experiments performed two or more times. Basal albumin promoter activity was set at 1.0 for each culture condition; the fold increase over basal transcription is shown by the bars for each multimer construct. In agreement with the results shown here for the eG dimer (construct 2), a trimer of the eG site was 40% less active in HepG2 cells on collagen than on plastic and was completely inactive in HeLa cells on plastic (data not shown). The autoradiographs show primer extensions of RNA from cells transfected on plastic (Pl.) or collagen (Col.); lane numbers correspond to plasmid numbers in panel a. Primer extension products are indicated as in Fig. 2; nonspecific products migrate between the arrowheads in the HeLa cell assay.

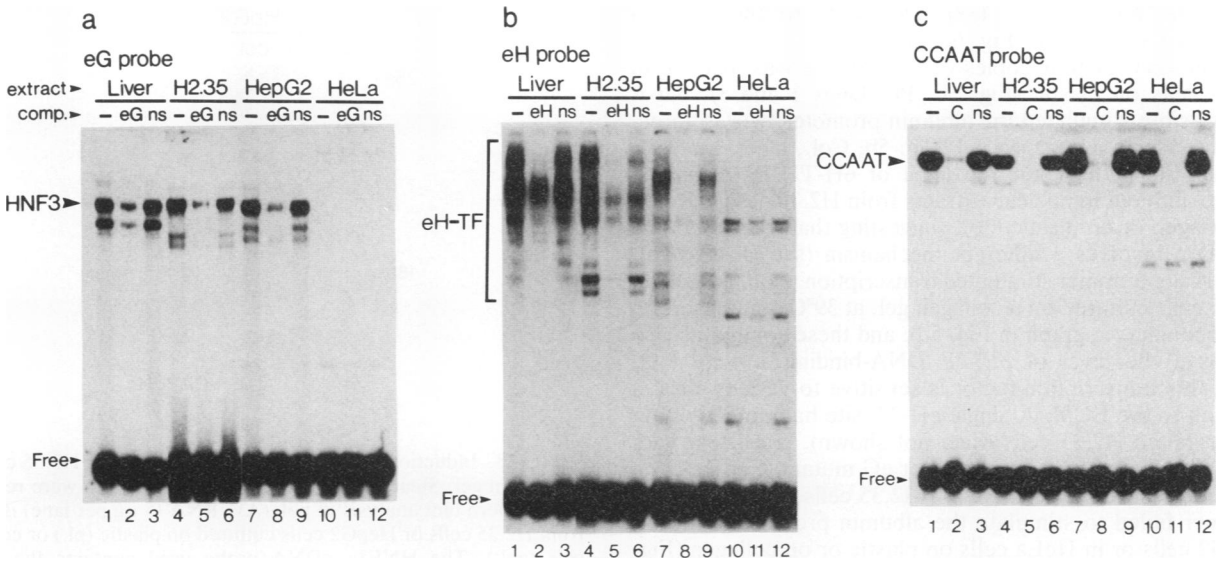

FIG. 6. Comparisons of HNF3 and eH-TF DNA-binding activities in hepatic and nonhepatic cell lines. Radioactive oligonucleotide probes of the eG site (a), eH site (b), or albumin promoter CCAAT site (c) were incubated with 7.5 μg of nuclear extract isolated from mouse liver (lanes 1 to 3), H2.35 cells on collagen at 33°C (a and c; lanes 4 to 6) or plastic at 39°C (b; lanes 4 to 6), HepG2 cells on collagen (lanes 7 to 9), or HeLa cells on plastic (lanes 10 to 12). A 100-fold molar excess of nonradioactive competitor oligonucleotide (comp.) was added as indicated: –, no competitor; eG and eH, HNF3 and eH-TF binding sites, respectively, from the albumin enhancer; C, albumin promoter CCAAT site; ns, nonspecific competitor containing either the CCAAT site (a) or the HNF1 site (b) from the albumin promoter, or the albumin eG site (c). Binding reactions were subjected to electrophoresis on native 8% polyacrylamide gels, and gels were dried and exposed to X-ray film. Specific HNF3, eH-TF, and CCAAT factor binding complexes are indicated at the sides of the gels. Free, free probe. The HNF3α and -β complexes from liver extracts comigrate as the upper band shown by the arrowhead; the HNF3γ complex is the lower band in the liver lanes (27). The H2.35 lanes in panel a are shown at twice the exposure of the other lanes.

the eG site was seen in HepG2 cells on collagen gels (Fig. 5c, Col., lanes 1 and 2). HepG2 cells cultured on collagen contained significantly more HNF3 binding activity than did H2.35 cells on collagen (Fig. 6a; H2.35 lanes are shown at twice the exposure time of HepG2 lanes). The specificity of HNF3 binding activities for the eG site in both HepG2 cells and liver extracts was confirmed by effective cross-competition with the HNF3 binding site from the TTR promoter (Fig. 7a, lanes 4, 9, and 14) (12, 27) and by the same pattern of binding and competition for a complex of the eG probe with an in vitro translation product of the cloned HNF3α cDNA (Fig. 7a, lanes 16 to 19) (26).

Interestingly, the level of HNF3 binding activity increased three- to fivefold when HepG2 cells were cultured on a collagen gel (Fig. 7a; compare lanes 6 and 7 with lanes 11 and 12). This result demonstrates that the matrix regulation of these factors is not specific to H2.35 cells. As a control, all extracts were shown to have comparable levels of the

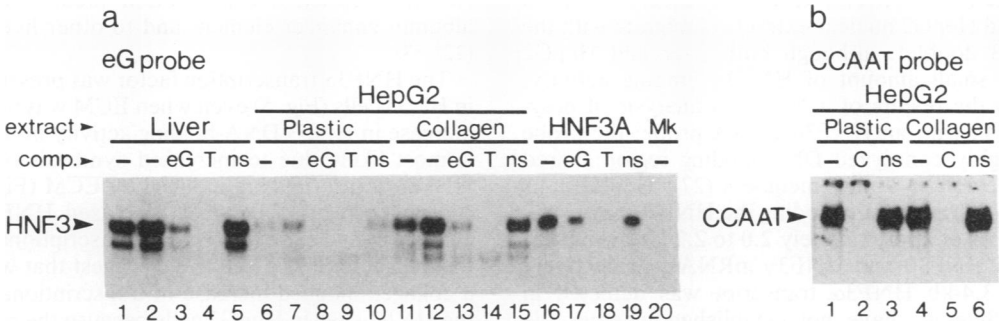

FIG. 7. Induction of HNF3 DNA-binding activity in nuclear extracts by culturing HepG2 cells on collagen. (a) Electromobility shift assays performed with the eG site probe as described for Fig. 6 but using 3 μg (lanes 1, 6, and 11) or 6 μg (lanes 2 to 5, 7 to 10, and 12 to 15) of nuclear extract from mouse liver (lanes 1 to 5), HepG2 cells on plastic (lanes 6 to 10), or HepG2 cells on collagen (lanes 11 to 15), or using an in vitro translation product of the cloned HNF3α gene (plasmid provided by Rob Costa; lanes 16 to 19, labeled HNF3A). In lane 20 (Mk), the eG probe was incubated with a mock in vitro translation reaction mixture lacking RNA. (b) Control assays using the albumin CCAAT site probe with 6 μg of nuclear extract from HepG2 cells on plastic (lanes 1 to 3) or collagen (lanes 4 to 6). T, competitor oligonucleotide containing the HNF3 site from the TTR promoter (12); ns, nonspecific competitor containing the HNF1 site from the albumin promoter (a) or the eG site from the albumin enhancer (b). Other competitors are as indicated in Fig. 6. Note that as seen previously (32), the TTR site is a more efficient competitor for the eG-binding activity in nuclear extracts as well as for eG binding by the HNF3α translation product. Specific binding complexes are indicated as in Fig. 6.

ubiquitous NF-Y factor (39) that binds to the albumin promoter CCAAT site (Fig. 6c and 7b).

A multimer of four copies of the eH site was inactive in H2.35 cells on plastic (Fig. 5b, Pl., lanes 1 and 3), but it reproducibly stimulated the albumin promoter two- to threefold in cells on a collagen gel (Fig. 5b, Col., lanes 1 and 3). Neither the pattern nor the level of eH-TF DNA-binding activity differed in nuclear extracts from H2.35 cells cultured on collagen or on plastic (30), suggesting that transcriptional induction involves a different mechanism (see Discussion). The eH site tetramer stimulated transcription about fivefold in H2.35 cells cultured on a collagen gel, at 39°C, and in serum-free medium (see graph in Fig. 5b), and these conditions also increased the level of eH-TF DNA-binding activity (30). Thus, this transcription factor is sensitive to various cues in addition to the ECM. A single eH-TF site had no activity in differentiated H2.35 cells (data not shown), consistent with the lack of activity of the enhancer eG mutant (Fig. 4).

In contrast to the results with H2.35 cells, the eH-TF site tetramer failed to stimulate the albumin promoter either in HepG2 cells or in HeLa cells on plastic or on collagen (Fig. 5c and d, lanes 1 and 3). Electromobility shift assays with an eH-TF site probe revealed a complex pattern of bands in all extracts tested (Fig. 6b), with the majority of bands in HeLa cells not specifically competable (lanes 10 to 12). There were different specific binding complexes in H2.35 and HepG2 cells, with a subset of complexes from each comigrating with complexes seen in liver extracts (Fig. 6b, lanes 1, 4, and 7). It appears that the lack of a matrix response in HepG2 cells, with either the albumin enhancer or the eH multimer, is due to differences in the eH site binding activities present in HepG2 and H2.35 cells; also, the H2.35 binding pattern more closely resembles the liver pattern. The nature of the different mobility shift complexes with the eH site probe is under investigation (21a). In summary, the ECM appears to coordinately regulate both HNF3 and eH-TF in H2.35 cells, and it is the binding of these factors together at the albumin enhancer that cooperatively stimulates transcription.

HNF3α mRNA levels are induced by ECM in H2.35 cells. We next sought to address whether HNF3 proteins were regulated by matrix at the level of activity or the level of synthesis. Electomobility shift complexes of HNF3α and HNF3β migrate as a closely spaced doublet, whereas the HNF3γ protein migrates faster (Fig. 6 and 7; 27). The major DNA-binding activities specific to the enhancer eG site in liver, H2.35, and HepG2 nuclear extracts comigrate with the HNF3α/HNF3β doublet, although both liver and HepG2 cells contain a small amount of HNF3γ binding activity. Figure 8 shows the results of a Northern analysis of polyadenylated RNAs with an HNF3α cDNA probe; the probe contains the highly conserved DNA-binding region shared among the three HNF3 family members (27). HepG2 cells contain the 3.4-kb transcript encoding the HNF3α protein as well as transcripts of approximately 2.0 to 2.2 kb, which are the sizes of the HNF3β and HNF3γ mRNAs, respectively (27); only the 3.4-kb HNF3α transcript was detected in H2.35 cells. While we have not established whether the matrix-inducible eG site-binding protein in HepG2 cells is HNF3α or HNF3β, or both, we conclude that in H2.35 cells the relevant protein is HNF3α.

HNF3α mRNA was induced three- to fourfold by culturing H2.35 cells on a collagen gel, when RNA levels were normalized to GAPDH mRNA used as an internal control (Fig. 8). The absolute levels of the HNF3 mRNAs in HepG2 cells were too low to quantitate induction by collagen, even though the cells had relatively high levels of DNA-binding activity. On the basis of our data from H2.35 cells, we conclude that an ECM gel substratum can cause a selective increase in HNF3α mRNA, DNA-binding activity, and transcriptional activation function, thereby contributing to hepatocyte differentiation.

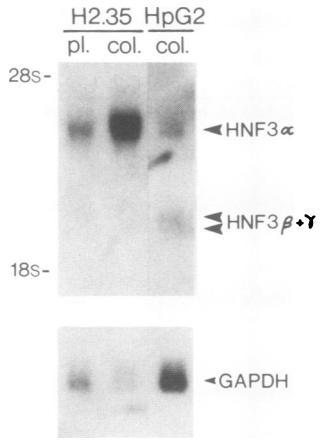

FIG. 8. Induction of HNF3α mRNA by culturing H2.35 cells on a collagen gel. Steady-state levels of HNF3 mRNAs were revealed by Northern blot analysis of poly(A)$^+$ RNA (5 μg per lane) isolated from H2.35 cells or HepG2 cells cultured on plastic (pl.) or collagen gels (col.). The HNF3α cDNA probe used contains the DNA-binding region that is highly conserved among members of the HNF3 family (28). The blot was subsequently rehybridized to a GAPDH cDNA probe. Positions of transcripts for HNF3α, HNF3β and -γ, and GAPDH, and of 18S and 28S rRNAs, are indicated.

DISCUSSION

The central focus of our studies has been an examination of how the extracellular environment influences endogenous factors to maintain cell differentiation. In this report, we addressed how the ECM promotes cell differentiation by studying albumin gene transcription, a function performed exclusively in the liver by hepatocytes. Transcription from both endogenous and transfected albumin genes was selectively activated by culturing a hepatocyte-derived cell line on ECM gels. This effect was mediated, at least in part, by two liver-enriched transcription factors that bind to the albumin enhancer element and to other liver-specific genes (12, 53).

The HNF3α transcription factor was present and functional in H2.35 cells (Fig. 5) even when ECM was not provided. The increase in HNF3 DNA-binding activity in response to ECM is most likely due to increased synthesis of HNF3α, since HNF3α transcripts are induced by ECM (Fig. 8). There was a clear correlation between increased HNF3 DNA-binding activity in nuclear extracts and transcriptional stimulation by the eG site in H2.35 cells. We suggest that we did not detect a collagen-induced increase in transcriptional stimulation by the HNF3 sites in HepG2 cells because the higher abundance of HNF3-binding proteins in these cells than in H2.35 cells (Fig. 6) saturated our expression assay (Fig. 5).

ECM activation of the albumin enhancer via the factor eH-TF was more complex. Our previous work showed that culture on an ECM gel was insufficient to increase the DNA-binding activity of eH-TF in H2.35 cells as assayed in nuclear extracts (30). However, multimers of the eH-TF binding site stimulated transcription in H2.35 cells cultured on an ECM gel (Fig. 5). Thus, ECM either causes eH-TF to

undergo a posttranslational modification that affects its ability to stimulate transcription (e.g., reference 22) or induces an unidentified factor that permits eH-TF to function. In either case, it appears that eH-TF and HNF3α are regulated by or mediate the effects of ECM by different mechanisms.

Single binding sites for HNF3 and eH-TF were totally unresponsive to matrix induction (14a). Thus, in the context of the albumin enhancer, and presumably at other regulatory sites, each factor stimulates transcription only in conjunction with the other, or with other factors. For example, the enhancer was not activated by matrix in HepG2 cells apparently because eH-TF was unable to mediate transcriptional induction. Although ECM only modestly increased the transcriptional activities of eH-TF or HNF3 site multimers, the coordinate induction of both factors in H2.35 cells was sufficient to switch the albumin enhancer from an inactive to an active state. This system exemplifies how a specific combination of distinct factors can activate a transcriptional regulatory element (51).

Malleable gel matrices of either type I collagen or basement membrane proteins, both of which could yield to cytoskeletal tensions, permitted H2.35 cells to assume a cuboidal shape and activated the enhancer, while rigid, nonyielding matrices of the same proteins did not. Since we used the same type I collagen and EHS protein stocks to prepare both gel and nongel substrata, it is unlikely that the induction of H2.35 cell differentiation by ECM gels was caused by contaminating factors in the matrix preparations. Instead, enhancer activity appeared dependent on the tensile properties of the ECM substrata and correlated with cell morphology. Other cell culture systems have also demonstrated that differentiated functions can depend on the mechanical properties of the ECM and apparently on cell shape. For example, culture of mammary epithelial cells on floating collagen gels induces both a cuboidal shape and the synthesis of a number of milk proteins; neither of these phenotypes was induced on collagen matrices that did not permit cell contraction (28). Similarly, cultured endothelial cells can be induced to differentiate into capillarylike structures by an ECM-derived substratum that does not restrict changes in cell shape (21). The same results were obtained with substrata composed of different ECM molecules, suggesting that, as with H2.35 cells, capillary development is influenced by the mechanical properties of the ECM and its ability to affect cell morphology.

A recent study showed that when primary rat hepatocytes were cultured on a rigid, type I collagen substratum and then exposed to EHS proteins diluted into the culture medium, transcription of the endogenous albumin gene was increased without induction of a cuboidal cell shape (8). We found that the transfected albumin enhancer was inactive in H2.35 cells cultured under the same conditions (data not shown), suggesting either that the relevant signal transduction pathways were not activated or that in the previous study, regulatory factors other than the ones we identified were responsive.

Changes in cell shape promoted by the ECM involve extensive cytoskeletal reorganization and altered cell-cell interactions (2, 6). We suggest that the albumin enhancer-binding factors may act as either direct or indirect recipients of such signals. Although the lack of response of eH-TF to collagen in HepG2 cells is consistent with the lack of dramatic morphological changes of these cells on collagen, the fact that HNF3-binding activity was increased by collagen in HepG2 cells suggests that morphological changes control, or are associated with, only a subset of the cellular responses to ECM.

In a recent report, 5′-flanking regions from the β-casein gene were shown to stimulate transcription in mammary epithelial cells in response to ECM and hormones (43), although distinct transcription elements that mediate this response have not yet been reported. It is likely that the induction of β-casein and other examples of ECM-promoted differentiation (33, 47) also involve regulation of cell-enriched transcription factors by ECM. Since both the stimulus and the responsive factors are now known for the albumin gene, it should be possible to work from both ends of the pathway to further define the mechanisms involved.

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