

Regulation of Rat Mammary Gene Expression by Extracellular Matrix Components

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In the mammary gland the induction and maintenance of differentiation are dependent on both lactogenic hormones and the extracellular matrix (ECM). Since mammary epithelial cells differentiate on a basement membrane *in vivo* we have examined the effects of basement membrane components on the expression of milk protein genes in primary rat mammary cultures. We examined the effects of a basement membrane gel derived from the Englebreth-Holm-Swarm tumor as well as its major component, laminin, on the expression of a group of milk protein genes. We demonstrate that the basement membrane gel induces α -casein and α -lactalbumin (α -LA) accumulation up to 160- and 70-fold, respectively, of that on tissue culture plastic. Laminin, a major component of the basement membrane, also caused significant induction of these same proteins. In order to determine whether these ECM effects occurred at a translational or post-translational level, pulse-chase experiments were performed. These experiments demonstrated that a laminin substratum selectively effects milk protein turnover and secretion. In order to demonstrate whether ECM effects occurred at the level of steady state accumulation of mRNA we performed dot blot and Northern analyses using cloned cDNA probes for α -, β -, and γ -caseins and α -LA. These studies demonstrated that ECM components induced α - and β -caseins up to 10-fold, and α -LA up to 3-fold, with no significant effect on γ -casein. These results demonstrate that milk protein genes are not coordinately regulated by ECM components. Furthermore, since the amount of induction of milk proteins exceeds the amount of induction of mRNAs for these proteins, we conclude that in our system a major effect of ECM components is at the translational and/or post-translational levels. Based on these findings we propose a model in which basement membrane components effect mammary gene expression at multiple levels. © 1987 Academic Press, Inc.

During development mammary epithelial cells undergo growth and differentiation which is characterized by the expression of a defined set of milk protein genes. *In vitro* studies have indicated that tissue-specific gene expression is influenced by hormonal factors, cell-cell interactions, and the extracellular matrix (ECM)² [1-4]. Using organ cultures it has been shown that lactogenic hormones including prolactin, insulin, and glucocorticoids modulate milk protein gene expression by effecting both mRNA transcription and mRNA turnover [5-7]. Studies of the effects of ECM on milk protein gene expression have primarily utilized stromal collagen gels as a substratum. These studies have shown that cells obtained from pregnant rabbit or mouse mammary gland express casein mRNAs and proteins when these cells are cultured on floating collagen

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² Abbreviations used: α -LA, α -lactalbumin; ECM, extracellular matrix; EHS, Englebreth-Holm-Swarm tumor.

gels [8–12]. It has been proposed that cell-shape changes associated with gel contraction may modulate this effect [13, 14]. Alternately, we and others have proposed that the effects of floating collagen gels might be indirectly due to the deposition of basement membrane components by mammary cells on these gels [2, 15–18].

We have previously demonstrated that a biomatrix extracted from pregnant rat mammary glands promoted the synthesis of α -lactalbumin (α -LA) by primary mammary cultures [2]. This biomatrix is composed of both stromal and basement membrane components. If the induction of mammary differentiation on floating collagen gels and biomatrix were due to basement membrane components associated with these systems, then one would predict that basement membrane components themselves would directly effect tissue-specific gene expression of mammary epithelial cells. *In vivo* these epithelial cells are directly associated with such a basement membrane which is composed of laminin, type IV collagen, entactin, heparan sulfate proteoglycans, and other glycoproteins [19, 20]. The integrity of the basement membrane is required for continued mammary cell growth [21, 22] and maintenance of differentiation [22]. Dissolution of this basement membrane is associated with mammary involution [23]. Based on these observations we have examined the effect of a basement membrane gel and an isolated basement membrane component, laminin, on the expression of milk protein genes in primary rat mammary cultures under serum-free hormonally defined conditions. We demonstrate that in the absence of stromal collagen these basement membrane components effect milk protein gene expression at both the mRNA and protein levels. While the mRNAs for the milk protein genes are affected by ECM components, there is a larger increase in the accumulation of milk proteins than can be accounted for solely by increases in mRNA accumulation. These experiments suggest that in this system a major effect of ECM components on tissue-specific mammary gene expression is at the translational and/or post-translational levels in addition to regulation at the mRNA level. The use of this *in vitro* culture system should enable us to examine the mechanisms by which milk protein gene expression is regulated by specific ECM components.

MATERIALS AND METHODS

Mammary cell preparation and culturing conditions. Mammary cells were isolated from perphenazine-stimulated virgin female Sprague–Dawley rats by limited collagenase digestion, differential filtration, and differential attachment to tissue culture plastic, as previously described [2]. The cells were plated at a density of 10^5 cells/cm² on 35-mm tissue culture dishes (Becton Dickinson, Oxnard, CA) coated with various substrates. Organoids were composed of small alveolar or ductal structures composed of 10–50 cells. The cultures were incubated at 37°C in a 5% CO₂ atmosphere. The cells were plated in Medium 199 supplemented with 5% fetal bovine serum, hydrocortisone 0.5 μ g/ml (Sigma Chemical Co.), estradiol-17 β 1 ng/ml (Sigma Chemical Co.), ovine prolactin 0.3 μ g/ml (Sigma Chemical Co.), insulin 0.1 μ g/ml (Eli Lilly and Co., Indianapolis, IN), and gentamicin 50 μ g/ml. The culture medium was changed 24 h after plating and replaced with a serum-free defined medium containing ovine prolactin 0.3 μ g/ml, insulin 5 μ g/ml (GIBCO Laboratories, Grand Island, NY), epidermal growth factor 10 ng/ml (Collaborative Research Inc., Lexington, MA), fetuin 1 mg/ml (Sigma Chemical Co.), hydrocortisone 0.2 μ M (Sigma Chemical Co.), and gentamicin 50 μ g/ml. Collagen gels were released to float 24 h after plating. The medium was changed every 3 to 4 days.

Preparation of substrata and laminin. Collagen gels were prepared from rat tail tendons as described [24]. The basement membrane gel was prepared from the Engelbreth-Holm-Swarm tumor as described by Kleinman *et al.* [25]. This gel has been shown to contain laminin, type IV collagen, entactin, nidogen, and heparan sulfate proteoglycan [26]. Laminin, the major ECM component of this gel, comprises 85% of the total protein. For some experiments, commercially prepared Matrigel (Collaborative Research Inc.) was utilized. Laminin was prepared as described by Timpl *et al.* [27] and its purity assayed by SDS-PAGE [28] and silver staining [29]. Laminin was stored at -70°C prior to use, diluted in water, and plated at $5\ \mu\text{g}/\text{cm}^2$ onto tissue culture dishes and allowed to dry.

Immunofluorescence microscopy. Epithelial and myoepithelial cell types were identified by indirect immunofluorescence using a mouse monoclonal antibody directed against the human milk fat globular membrane protein PAS-1 generously provided by Gordon Parry. This antibody stains the apical surface of mammary epithelial cells [30]. Myoepithelial cells were identified by staining with an affinity purified rabbit anti-type IV collagen antibody [22]. Mammary cells on laminin-coated ($5\ \mu\text{g}/\text{cm}^2$) or uncoated tissue culture chamber slides (Miles Scientific, Naperville, IL) were fixed with 3.7% formaldehyde/0.1% glutaraldehyde in PBS after varying lengths of time in culture, washed with PBS, and incubated with anti-PAS-1 at a 1:10 dilution for 1 h. All antibodies were diluted in PBS with 0.1% bovine serum albumin (BSA). After washing with PBS, the slides were incubated with a fluorescein-conjugated goat anti-mouse (u chain) antibody (CooperBiomedical, Malvern, PA) for 1 h. The slides were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS, washed with PBS, and incubated with anti-type IV collagen antibody for 1 h. Rhodamine-conjugated sheep anti-rabbit IgG was used as a second antibody. All procedures were performed at room temperature. For control experiments, PBS/0.1% BSA was substituted for the primary antibody with negligible staining. The slides were photographed on a Leitz epifluorescence microscope.

Measurement of α -lactalbumin. Medium from cells cultured on various substrata was collected from 4 to 6 35-mm dishes over the course of 2 weeks. The medium was centrifuged at 2000g for 5 min at 4°C and stored at -20°C . A radioimmunoassay on 4 to 6 replicate samples was performed as we have described [2] using purified rat α -LA and rabbit anti-rat α -LA (E. G. and G. Mason Research Institute, Worcester, MA). The data were analyzed by a one way analysis of variance to assess both variation within an individual experiment and among a series of experiments.

Cell layers were washed three times with 0.14 M NaCl, 50 mM Tris-HCl, pH 7.4, and then lysed with 25 mM Tris-HCl, 50 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, pH 7.4, with Aprotinin 200 units/ml (Boehringer-Mannheim, Indianapolis, IN). Plates were scraped with a rubber policeman, the gels were minced, and the extract was gently vortexed. The lysate was centrifuged at 10,000g for 30 min and the supernatants were removed. All supernatants were brought to an equal volume with the lysis buffer and stored at -70°C . All procedures were performed at 4°C . Aliquots were assayed for α -LA by radioimmunoassay.

Cells numbers were measured by removing the cells on plastic- or laminin-coated dishes with 0.25% trypsin/0.1% EDTA (K. C. Biological, Lenexa, KS) and counting by hemocytometer. Collagen gels were first digested with collagenase 3 mg/ml (Sigma Chemical Co.) for 60 min at 37°C , and the cells were dissociated with 0.25% trypsin/0.1% EDTA and counted by hemocytometer. Basement membrane gels were first digested with 5% trypsin/0.5% EDTA at 37°C , and then cells were counted by hemocytometer.

Immunoblotting. An acid-precipitable fraction of skimmed rat milk was prepared and used to generate a rabbit polyclonal antibody as described by Lee *et al.* [10]. Rat anti-mouse monoclonal antibodies against α -casein and γ -casein were generously provided by Frank Stockdale [3]. A monoclonal antibody against rat β -casein was kindly provided by Charlotte Kaetzel [31]. Rabbit anti-rat transferrin was obtained from CooperBiomedical. Immunoblotting was performed as previously described [32].

Immunoprecipitation of milk proteins. Cultures were washed with methionine-free medium four times over 90 min at 37°C , and then labeled with $100\ \mu\text{Ci}/\text{ml}$ [^{35}S]methionine [$>1000\ \text{Ci}/\text{mmol}$] for 24 h. In some cases, 3-h labeling experiments were performed with similar results. The longer period of labeling permitted briefer film exposures. The medium was removed, and deoxycholate and Nonidet P-40 were added to 1 and 0.5%, respectively. The cell layers were prepared as described above. Samples were stored at -70°C until assayed. TCA precipitation was performed on duplicate aliquots from each sample of media or cell layer fraction. A comparable number of TCA-precipitable counts was immunoprecipitated using preformed immune complexes by a modification of the method of Terhorst *et al.* [33] and Tolleshaug *et al.* [34]. Preformed immune complexes were prepared using 200 μl of buffer A (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), 200 μl of sheep anti-rabbit IgG, 12.0 mg/ml antibody protein (CooperBiomedical Inc.), and 200 μl of rabbit anti-rat milk antibody (positive at greater than 1:1000 by immunoblot) or 200 μl of preimmune rabbit

serum. The reaction mixture was incubated at room temperature for 30 min, and then stored for 1–2 days at 4°C. The complexes were precipitated by centrifugation at 500g for 2 min at 4°C, washed two times in buffer A, and then resuspended in 0.5 ml of buffer A. The [³⁵S]methionine-labeled medium or cell layer lysates were incubated with the preformed preimmune complexes for 30 min at 4°C on a rocking platform. Precipitates were collected by centrifuging at 12,000g for 10 min. The supernatants were incubated with the preformed immune complexes for 2 h at 4°C on a rocking platform. The entire sample or preimmune pellet, resuspended in 0.5 ml of buffer A, was layered on a sucrose step gradient composed of 40% sucrose, 10 mM Tris, pH 8.0; 30% sucrose, 0.2% Nonidet P-40, 10 mM Tris, pH 8.0; 20% sucrose, 0.5 M NaCl, 0.2% Nonidet P-40, 10 mM Tris, pH 8.0; and 10% sucrose, 0.5% Nonidet P-40, 10 mM Tris, pH 8.0, and centrifuged at 30,000g for 30 min at 4°C. The pellets were washed once in buffer A and boiled in SDS-PAGE reducing buffer for 5 min. Samples were electrophoresed on 13.5-cm 10% slab gels, treated with En3Hance (Dupont New England Nuclear, Boston, MA), dried, and exposed at -70°C to Kodak XAR-5 film. Densitometry (Hoefer GS300) was performed in the linear range of exposure for the film and the areas under the peaks were integrated. To assure that this immune complex immunoprecipitation method was sensitive to quantitative differences in the antigen, we used a range of sample volumes for each condition.

Pulse-chase experiments. Equivalent number of cells on tissue culture plastic or laminin-coated dishes were incubated in methionine-free medium for 1 h, and then pulsed with 200 µCi/ml [³⁵S]methionine for 1 h. Similar results were obtained with a 30-min labeling. However, in order to detect a signal on tissue culture plastic dishes, the longer labeling period was utilized. Cultures were washed once quickly with medium supplemented with 4 mg/ml methionine (Sigma Chemical Co.), and then chased with medium containing 4 mg/ml methionine for the indicated times. Medium and cell layers were harvested, immunoprecipitated, electrophoresed, dried, and analyzed as described above. Additionally, the bands were cut from the filter, dissolved in H₂O₂ at 100°C for 2 h, solubilized in Beckman Ready-Solv-MP, and counted in a scintillation counter.

RNA isolation. Total cytoplasmic RNA was isolated from cultured cells according to procedures described by Maniatis [35] with the following modifications. Detergent lysis of cells was accomplished by the addition of lysis buffer [50 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 1% Nonidet P-40, and RNasin 1 mg/ml (Promega, Madison, WI)] to cells in the culture dish following the removal of medium and several rinses of cells in PBS. After lysis and removal of cellular debris and nuclei by centrifugation at 12,000g, the cytoplasmic extract was digested with proteinase K (0.2 mg/ml) (Boehringer-Mannheim). Following phenol extraction, cytoplasmic RNAs were precipitated in 0.3 M sodium acetate and 95% ethanol. The cytoplasmic RNA was subsequently treated with RNase-free DNase (Promega) to remove any contaminating DNA sequences homologous to milk protein RNAs, phenol extracted, and precipitated as described. Yield of total cytoplasmic RNA was measured by spectrophotometry.

Measurement of steady state levels of milk protein cytoplasmic RNAs. The levels of mRNAs for the milk protein genes were measured by RNA dot blot analysis using ³²P-labeled cDNA probes for these genes. The plasmid cDNA clones for α -, β -, and γ -caseins were generously provided by Jeffrey Rosen and have been extensively characterized [36, 37]. A mouse cDNA clone of α -LA was provided by M. R. Banerjee. A plasmid DNA clone, designated pA, containing the human 28 S ribosomal gene was a gift of Jerry Gorski. Using the appropriate restriction endonuclease, the cDNA insert was excised from the plasmid DNA and isolated by gel electrophoresis and electroelution. The resulting cDNA inserts are as follows: α -casein, 1.060 kb (*Pst*IHpaII); β -casein 0.5 and 0.6 kb (*Pst*I); γ -casein, 0.9 kb (*Pst*I); α -LA, 0.6 kb (*Pst*I); and pA, 1.4 kb (*Bam*HI) [38]. Approximately 100 ng of cDNA insert was labeled by nick-translation resulting in a specific activity of 3×10^8 cpm/µg of DNA for milk protein DNA probes and 2×10^7 cpm/µg of DNA for 28 S ribosomal DNA.

Dot blot assays of RNAs isolated from primary cell cultures were performed using four serial dilutions from 0.25 to 2.0 µg [39]. RNA was isolated from 4-day lactating rat mammary glands using a guanidinium isothiocyanate/CsCl method [40] and dotted on the filters in six serial dilutions from 0.15 to 0.5 µg. Filters were prehybridized and then hybridized in 50% formamide, 2×Denhardt's, 5×SSPE, 0.1 mg/ml salmon sperm DNA, and 0.1% SDS at 44°C for 20 h. Following hybridization, filters were washed first in 2×SSC, 0.1% SDS at 22°C, then in 0.1×SSC and 0.1% SDS at 50°C, followed by washes in 0.1×SSC, 0.1% SDS at 22°C. The steady state levels of cytoplasmic RNA for each milk protein were measured by graphing the intensity of RNA dots as measured by densitometry versus serial dilutions of cytoplasmic RNA. The slope of the linear curve was calculated and used to quantitate the amount of increase in RNA steady levels by normalizing to tissue culture plastic. Similar results were obtained by quantitating the signal by scintillation counting. RNA samples were also size fractionated on formaldehyde-agarose gels, transferred to nitrocellulose [35], and hybridized to ³²P-labeled α -casein cDNA.

RESULTS

Morphologic Effects

We examined the effects of different substrata on the morphology of primary rat mammary cultures. Cells on tissue culture plastic or laminin-coated dishes appeared flattened at the light microscopic level. However, cells plated on the laminin substrate developed refractile lipid vacuoles, an appearance consistent with the expression of differentiated function [13, 14]. Cells on tissue culture plastic failed to achieve this appearance (compare Figs. 1*a* and 1*b*). Cells on released collagen gels also developed these vacuoles (Fig. 1*c*) as well as the characteristic cuboidal morphology seen at the electron microscopic level (data not shown). This appearance accompanied gel contracture. Cells plated on the basement membrane gel assumed a globular appearance with large vacuoles (Fig. 1*d*). This occurred in the absence of any detectable gel contracture and was seen as early as 3 days in culture.

Effects of Extracellular Matrix on Mammary Gene Expression

These morphologic observations suggested that matrix components promoted the differentiation of mammary epithelial cells. In order to characterize these findings further, we examined the effects of a basement membrane gel and its major component laminin, and collagen gel on milk protein gene expression at the protein and mRNA levels. We further studied the temporal course of the induction of these milk proteins and mRNAs in culture over a 2-week period to determine if the effects of matrix components were direct and immediate or required prolonged time in culture.

Accumulation and Secretion of Milk Proteins

A polyclonal antibody directed against rat milk was utilized to immunoprecipitate milk proteins synthesized by primary rat cultures. Figure 2 is an immunoblot which demonstrates that this polyclonal antibody can detect α -casein (41–43 kDa), β -casein (28 kDa), and γ -casein (a series of bands between 18 and 22 kDa in rat milk). In addition, this antibody identified transferrin which was confirmed by immunoblotting milk with anti-rat transferrin (not shown). Xanthine oxidase (150 kDa) and butyrophilin (67 kDa), components of the milk fat globular membrane are also detected by this antibody in addition to 32 and 16 kDa proteins.

A representative set of immunoprecipitates of [³⁵S]methionine-labeled medium from cultures maintained on laminin, collagen gel, or the basement membrane gel using this antibody is shown in Fig. 3 and reveals two major bands, transferrin and α -casein. β - and γ -caseins were not detected by immunoprecipitation (Fig. 3) or by immunoblots utilizing monoclonal antibodies against these proteins (data not shown).

The results of several such immunoprecipitation experiments are quantified in Figs. 4*a* and *b* and are represented as amount of induction relative to that on tissue culture plastic. Total synthesis representing the sum of α -casein in the cell layer and medium was maximal on the basement membrane gel at all time points tested and reached a maximum of 160-fold relative to that on plastic by Day 14.

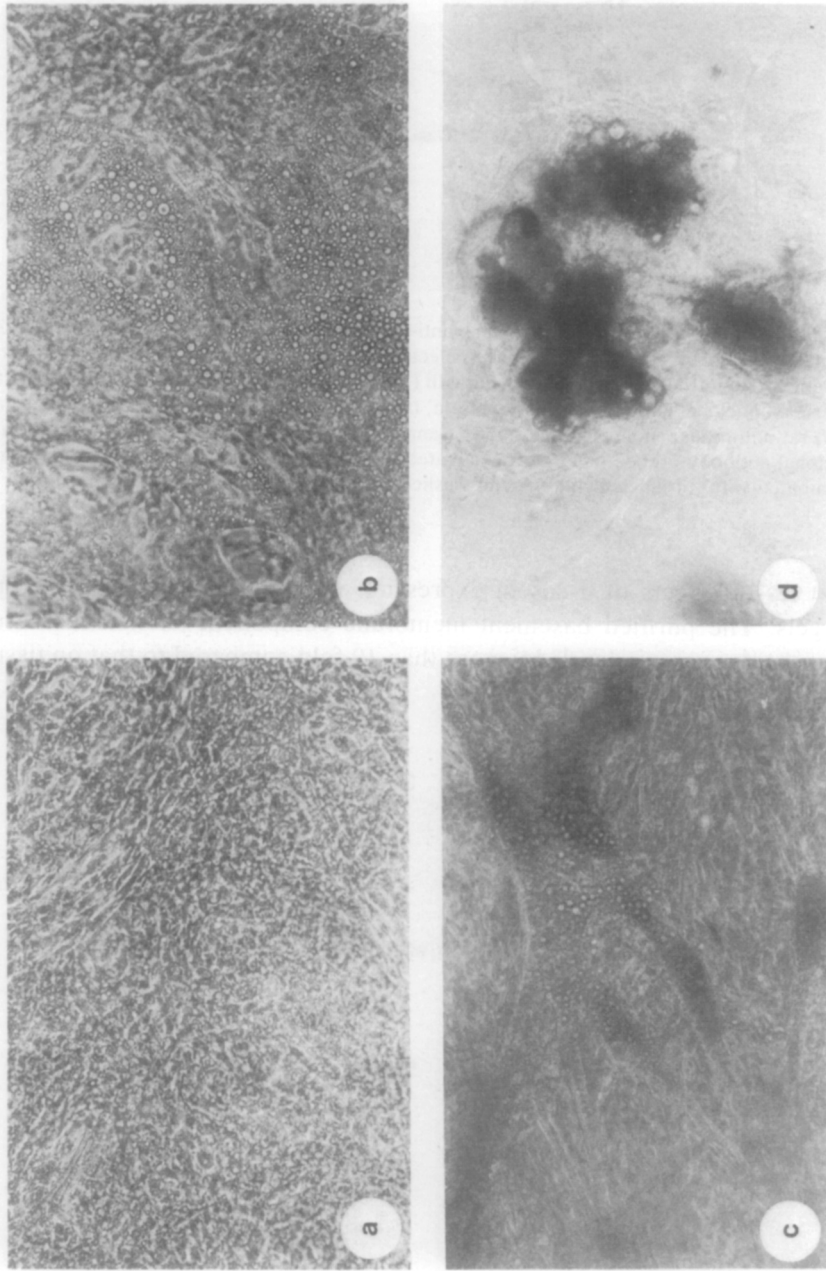


Fig. 1. Morphology. Mammary cells were maintained in culture for 2 weeks on (a) tissue culture plastic, (b) laminin-coated plastic, (c) floating collagen gel, and (d) EHS gel. Phase contrast micrographs were taken at 100 \times magnification.

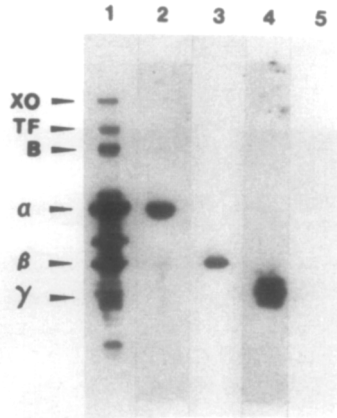


Fig. 2. Identification of proteins detected by the anti-rat milk antibody by western blot analysis. An acid-precipitable fraction of skimmed rat milk was electrophoresed on a 10% SDS-PAGE, transferred electrophoretically to nitrocellulose, and probed with (1) polyclonal rabbit anti-rat milk antibody, (2) rat anti-mouse monoclonal antibody against α -casein, (3) mouse anti-rat monoclonal antibody against β -casein, (4) rat anti-mouse monoclonal antibody against γ -casein, and (5) preimmune rabbit serum. The monoclonal antibody-probed lanes were incubated with a staph A-binding second antibody and all were incubated with ^{125}I -labeled staph A and washed and an autoradiogram was obtained.

Somewhat lesser effects of α -casein expression were seen for cells on floating collagen gels. The purified basement membrane component laminin increased total steady state α -casein levels by more than 10-fold compared to that on tissue culture plastic.

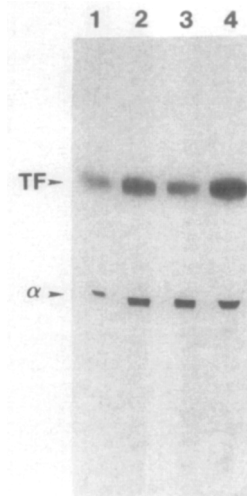


Fig. 3. Immunoprecipitation of [^{35}S]methionine-labeled medium. Equivalent TCA precipitable counts of [^{35}S]methionine-labeled medium from Day 3 cultures on (1) tissue culture plastic, (2) laminin-coated plastic, (3) released collagen gels, and (4) basement membrane gel-coated dishes were harvested, immunoprecipitated, electrophoresed, and dried as described under Materials and Methods and a fluorogram was obtained. TF, 80-kDa transferrin; and α , 43-kDa α 1-casein.

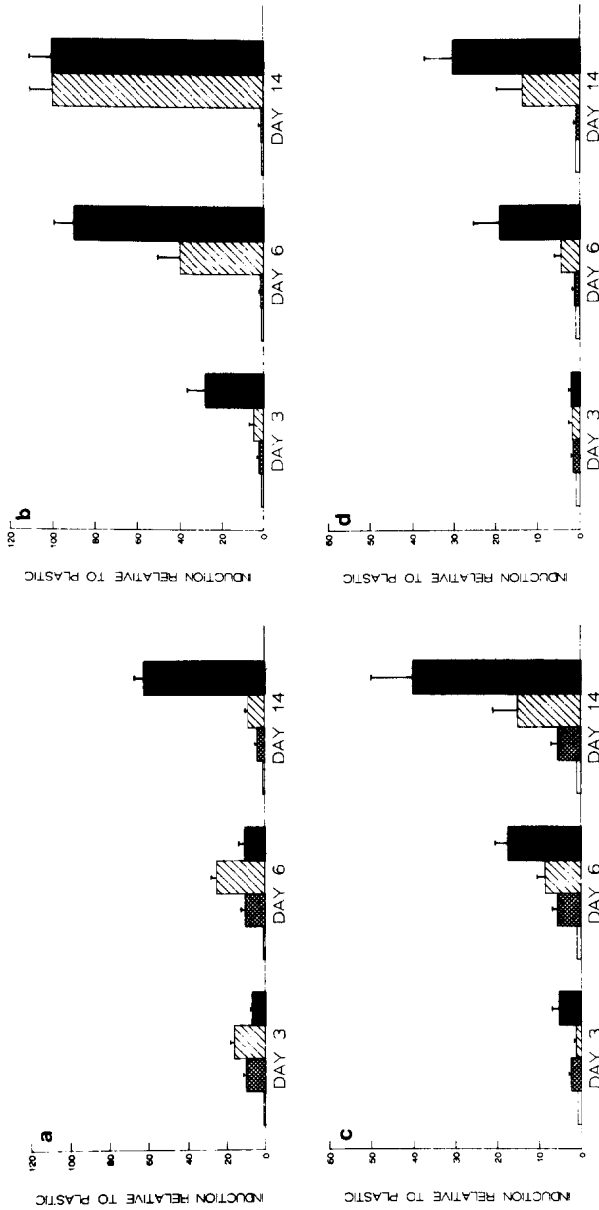


Fig. 4. α -Casein and α -lactalbumin synthesis. The mean amount of induction of α -casein relative to that of cells on plastic was determined by integrating densitometric scans of fluorograms from 2 to 6 experiments in (a) medium and (b) cell layer. The mean amount of induction of α -LA synthesis relative to that of cells on plastic from 2 to 11 experiments based on a radioimmunoassay and normalized for cell number in (c) medium and (d) cell layer. Error bars indicate the standard error of the mean. □, Tissue culture plastic; ▨, laminin; ■, Matrigel (basement membrane gel). (a) α -Casein—secreted, (b) α -Casein—secreted, cell layer. (c) α -Lactalbumin—secreted, (d) α -Lactalbumin—secreted, cell layer.

Effects of ECM components were apparent by Day 3 in culture but the amount of induction relative to that on tissue culture plastic increased progressively with time in culture. Although accurate assessment of secretion on gelled matrices is complicated by trapping of medium within the gel, it is apparent that the effects of ECM on α -casein production are not merely due to increased secretion. This is the case because cultures on ECM substrata had increased α -casein in both the cell layer and the medium.

α -LA was measured by radioimmunoassay and the results of several experiments are summarized in Figs. 4 *c* and 4 *d*. The effects of ECM were apparent by Day 3 in culture and increased with time. α -LA was maximally induced by the basement membrane gel reaching 70-fold compared to that on tissue culture plastic by Day 14. This effect was over two times greater than that on floating collagen gels. Laminin also had a significant although lesser effect on α -LA production reaching sevenfold greater than that on tissue culture plastic by Day 6. As was the case for α -casein, the induction of α -LA by ECM represents increased total production since milk protein is induced in both cell layer and medium on ECM substrata compared to that on tissue culture plastic. Furthermore, the increases in α -LA relative to that on tissue culture plastic with time in culture represent induction on matrix substrata rather than progressive dedifferentiation on tissue culture plastic. This was the case because the absolute values of α -LA as determined by radioimmunoassay remained 3 ng/10⁵ cells on tissue culture plastic. In contrast, on the basement membrane gel, α -casein production increased from 7 ng/10⁵ cells on Day 3 to 200 ng/10⁵ cells on Day 14.

On the basis of these data we postulated that the induction in steady state levels of milk protein accumulation on ECM components could be due to changes in milk protein turnover and/or differences in the steady state accumulation of milk protein mRNAs. To test this, we performed pulse-chase analysis of metabolically labeled immunoprecipitated milk proteins and RNA dot blot analysis.

Kinetics of α -Casein and Transferrin Synthesis

Pulse-chase experiments were performed to determine if the reduced levels of α -casein on tissue culture plastic resulted from differences in α -casein turnover. The results of a representative experiment are shown in Figs. 5 and 6 *a*. α -Casein in rat milk has been described as a doublet when examined by SDS-PAGE and by DEAE-cellulose chromatography [31]. $\alpha 1$ and $\alpha 2$ caseins have a high degree of structural homology in that they are both detected by a panel of five monoclonal antibodies [31]. It is not known if these two proteins share a precursor-product relationship or whether these differences are secondary to variable glycosylation or phosphorylation. However, only one mRNA species for α -casein has been described [36]. Pulse-chase experiments demonstrated α -casein as a doublet in the cell layer. In our electrophoretic system, these two proteins have an apparent molecular weight of 43 ($\alpha 1$) and 41 ($\alpha 2$) kDa. $\alpha 1$ -Casein is the predominant secreted species in both pulse-chase and steady state experiments (see Figs. 3, 5 *b*, and 5 *e*). In the steady state experiments summarized in Fig. 4 *b*, the predomi-

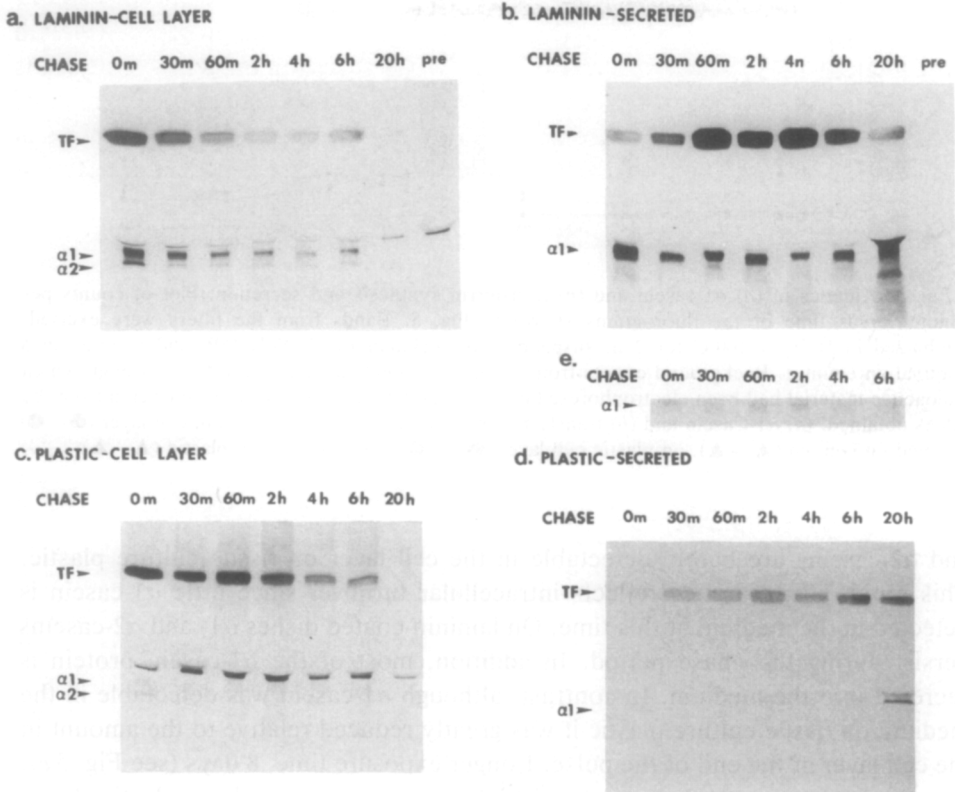


Fig. 5. Pulse-chase immunoprecipitation of α -casein and transferrin. Day 6 cultures on laminin (*a, b*) or plastic (*c-e*) were incubated for 1 h in methionine-free medium, pulsed with 200 μ Ci/ml [35 S]methionine for 1 h (0 min chase) at 37°C, washed once with medium containing an excess of unlabeled methionine, and then chased for the indicated times (m, minutes and h, hours, pre, a preimmune serum precipitated control). All samples were incubated with preimmune preformed complexes, and then incubated with the immune preformed complexes as outlined under Materials and Methods. The major band detected in the preimmune control was actin (45 kDa) as determined by immunoblotting with rabbit anti-actin antisera generously provided by S. Brown (not shown). This band is also detected to varying degrees in the immunoprecipitated samples as shown in *a-d*. Cell layers (*a* and *c*) and medium (*b* and *d*) from six 35-mm dishes were pooled for each time point, immunoprecipitated, electrophoresed, and dried and fluorograms were obtained. (*a-d*) 2-Day film exposures. Longer (8-day) film exposure (*e*) was required to detect the secreted α -casein from cells on plastic dishes.

nant α -casein species was $\alpha 1$ although in some cases $\alpha 2$ -casein was also detected in the cell layer on the basement membrane gel.

Following a 1-h labeling period, the total accumulation of $\alpha 1$ -casein is 27-fold greater on laminin-coated dishes compared with that on tissue culture plastic. This represents an increase both in the cell layer and in the medium during the period of the pulse (see Figs. 5*a-d*, 0-min chase) and is determined both by densitometric quantitation and by scintillation counting of the $\alpha 1$ -casein bands. This difference at the end of the pulse might reflect a rapid turnover of $\alpha 1$ -casein and/or decreased synthesis on tissue culture plastic. After a 30-min chase, $\alpha 1$ -

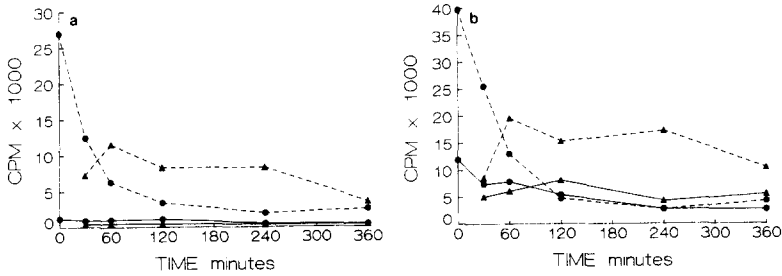


Fig. 6. Kinetics of (a) $\alpha 1$ -casein and (b) transferrin synthesis and secretion. Plot of counts per minute versus time of the fluorograms shown in Fig. 5. Bands from the filters were excised, solubilized in H_2O_2 at $100^\circ C$ for 2 h, suspended in Beckman Ready-Solv-MP, and counted in a scintillation counter. Background counts from a region of identical size of the filter through which radioactive material had been electrophoresed but without detectable band were subtracted from the values obtained. (a) $\alpha 1$ -Casein and (b) transferrin show the kinetics in the laminin cell layer (●—●) secreted on laminin (▲—▲), on plastic cell layer (●—●), and secreted on plastic (▲—▲).

and $\alpha 2$ -caseins are barely detectable in the cell layer on tissue culture plastic. This rapid disappearance reflects intracellular turnover since little $\alpha 1$ -casein is detected in the medium at this time. On laminin-coated dishes $\alpha 1$ - and $\alpha 2$ -caseins persist during the chase period. In addition, most of the $\alpha 1$ -casein protein is secreted into the medium. In contrast, although $\alpha 1$ -casein was detectable in the medium on tissue culture plastic it was greatly reduced relative to the amount in the cell layer at the end of the pulse. Longer exposure time, 8 days (see Fig. 5 e), was required to reveal the secretion of $\alpha 1$ -casein on tissue culture plastic. From these experiments we conclude that laminin decreases the intracellular turnover of $\alpha 1$ - and $\alpha 2$ -caseins, increases the secretion of $\alpha 1$ -casein, and may, in addition, increase synthesis of this protein. All of these factors together contribute to the increase of the steady state accumulation of these proteins on a laminin substratum.

The kinetics of transferrin synthesis and secretion were also examined (Figs. 5 and 6 b). Steady state immunoprecipitation experiments had demonstrated that transferrin synthesis and secretion were reduced on tissue culture plastic relative to matrix-coated dishes (see Fig. 3). Pulse-chase experiments indicate that at the end of the 1-h labeling period, the total accumulation of transferrin is two- to threefold greater on laminin-coated dishes compared with that on tissue culture plastic. Moreover, these pulse-chase studies indicate that less of the protein is secreted into the medium on tissue culture plastic than on laminin-coated substrata.

The effects of laminin on protein secretion for both α -casein and transferrin do not merely reflect an increase in general protein secretion for cultures on laminin substrata. This is the case because total protein secretion as measured by TCA-precipitable counts in the medium is equivalent on laminin and tissue culture plastic dishes (data not shown). Taken together, these data suggest that the basement membrane component laminin can selectively effect tissue-specific gene expression by modulating protein synthesis, turnover, and secretion.

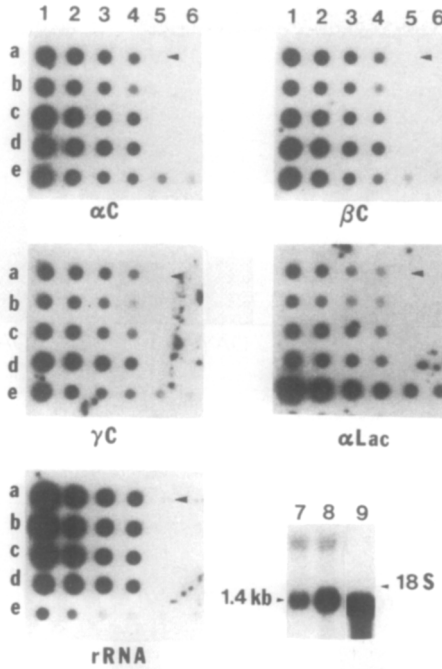


Fig. 7. Dot blots of cytoplasmic RNAs. Cytoplasmic RNAs isolated from primary rat mammary cells cultured on various substrata were dotted onto nitrocellulose filters and hybridized to ^{32}P -labeled cDNA probes. Representative dot blots for Day 6 are shown. Lanes (a) tissue culture plastic, (b) laminin coated plastic, (c) collagen gel, (d) basement membrane gel, and (e) lactating RNA. Cytoplasmic RNAs were serially diluted as follows. Lanes (1) 2.0 μg , (2) 1.0 μg , (3) 0.5 μg , (4) 0.25 μg . Lactating RNAs were serially diluted as follows. Lanes (1) 0.5 μg , (2) 0.25 μg , (3) 0.125 μg , (4) 0.062 μg , (5) 0.031 μg , (6) 0.015 μg . Northern blot of equal amounts of cytoplasmic RNA and lactating RNA is as follows: lanes (7) tissue culture plastic, (8) laminin-coated plastic, and (9) lactating RNA. The Northern blot was probed with ^{32}P -labeled α -casein cDNA. Yeast tRNA was dotted on each filter in a 2- μg aliquot as indicated by the arrowhead.

Steady State Levels of mRNA

Differences in the steady state accumulation of milk proteins on ECM components could be a reflection of different rates of synthesis in addition to differences in turnover. To determine if the increase in α -casein and α -LA protein accumulation could be accounted for by a proportional increase in the mRNAs for these two proteins, we performed dot blot analysis on RNA isolated from cells plated on laminin, collagen, or basement membrane gel. In addition, we probed the RNA extracted from these cultures for β - and γ -casein to determine if the reason for the failure to detect these proteins in mammary cultures was due to the lack of expression of the mRNAs for these proteins. Figure 7 shows a representative set of dot blots probed with ^{32}P -labeled cDNAs for α -, β - and γ -casein and α -LA and the results of several such experiments quantitated by densitometry are shown in Fig. 8. Northern blot analysis (Fig. 7, lanes 7–9) for α -casein confirmed the dot blot data and demonstrated a 3- to 10-fold induction on laminin relative to plastic. Northern blot analysis also indicated an intact 1.4-kb mRNA [37]. As can be

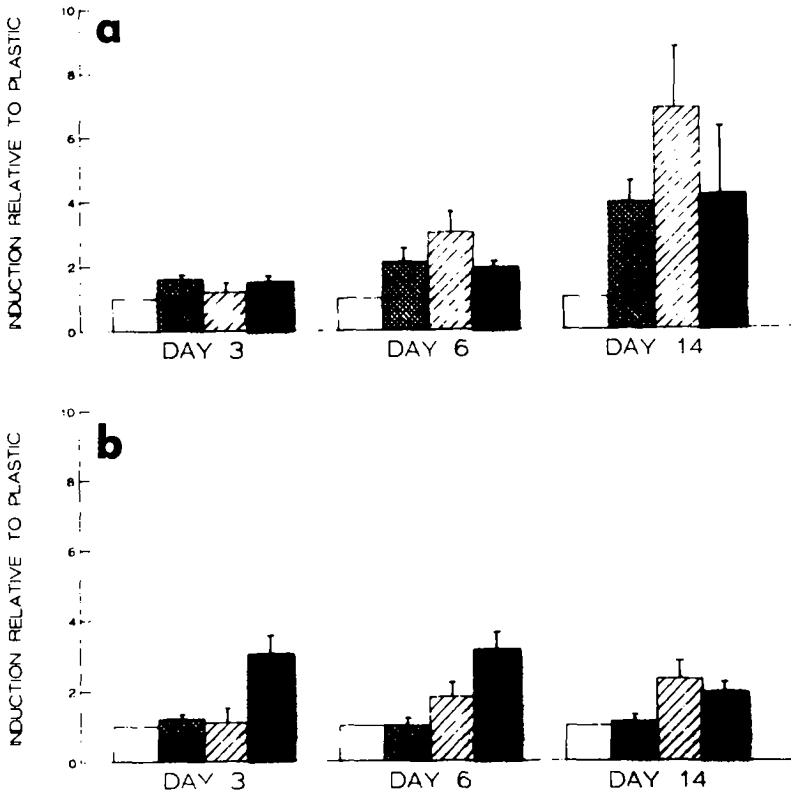


Fig. 8. (a) α -Casein and (b) α -lactalbumin mRNA accumulation. The mean amount of induction of (a) α -casein and (b) α -lactalbumin mRNA relative to that on plastic based on two to six dot blot filter hybridization experiments is shown. Error bars indicate the standard error of the mean. \square , plastic; \boxtimes , laminin; \blacksquare , released collagen gels; \blacksquare , Matrigel (basement membrane gel).

seen, cytoplasmic mRNAs for the various milk proteins are differentially regulated by ECM substrata. Steady state levels of α - and β -casein mRNAs accumulated up to fivefold more on basement membrane substrata than on tissue culture plastic while α -LA RNA levels under these same conditions were twofold greater than those on tissue culture plastic by Day 14. In contrast, there was no consistent induction of γ -casein mRNA by ECM substrata. Laminin substrata increased the steady state levels of α -casein mRNA and the inductive profile of accumulation was nearly comparable to that obtained on a basement membrane gel. The induction of milk protein mRNAs was similar on stromal collagen gels and basement membrane substrata.

The increases in milk protein mRNA levels detected on matrix substrata are not the result of global changes in total RNA or general mRNA synthesis. Ribosomal RNA accumulation (Fig. 7) remains essentially constant on all of the substrata tested. In addition, the increase in milk protein mRNA synthesis on matrix substrata does not represent a general increase in mRNA synthesis because actin mRNA, as detected by dot blot analysis, was maximal on tissue culture plastic dishes (data not shown).

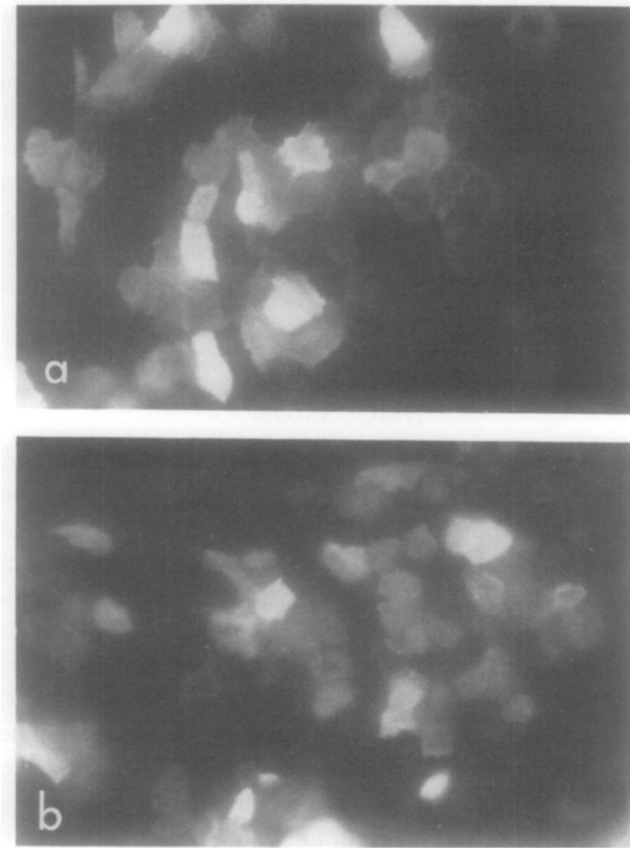


Fig. 9. Identification of epithelial cells. Mammary cells were maintained in culture for 2 weeks. Indirect immunofluorescence was performed using a mouse monoclonal antibody against human PAS-1. (a) Cells on a laminin-coated slide, (b) cells on an uncoated tissue culture plastic slide. 630 \times .

From these studies we conclude that although matrix components regulate the steady state levels of milk protein mRNAs, these effects do not account for the large induction of milk protein accumulation found on ECM substrata. For both α -casein and α -LA the amount of induction of these proteins by ECM far exceeds the amount of induction of their mRNAs.

Cell Types

We have demonstrated that culturing mammary cells on a laminin substratum increases the expression of several milk proteins and their mRNAs. These primary cultures of mammary cells are composed of a mixed population of epithelial and myoepithelial cells. The effect of laminin on milk protein gene expression might be due to a direct effect on the cells themselves or to an indirect effect resulting from the selection of a particular cell type. To determine if culturing on laminin altered the distribution of cell types, we performed indirect immunofluorescence utilizing the luminal epithelial cell marker PAS-1, a 200-kDa sialoglycoprotein of the milk fat globular membrane [30, 41]. Figure 9 demon-

strates that mammary cells cultured on either tissue culture plastic or laminin demonstrate similar immunofluorescent staining for PAS-1. Double labeling experiments using anti-PAS-1 and anti-type IV collagen, a marker of myoepithelial cells [42], were also performed on Day 3 and 6 cultures and revealed a similar distribution of epithelial cells and myoepithelial cells, on both tissue culture plastic and laminin (data not shown). These studies demonstrate that the failure to differentiate on tissue culture plastic is not due to the absence of luminal epithelial cells. Furthermore, culturing mammary cells on a laminin-coated substratum does not alter the distribution of epithelial or myoepithelial cells compared with those on tissue culture plastic.

DISCUSSION

It is now clear in a variety of systems that tissue-specific gene expression is influenced by the ECM. Although the molecular mechanisms involved in this regulation are unknown there is evidence that this occurs at multiple levels [43–45]. We have utilized the rat mammary gland as a model system to examine the effects of ECM components on milk protein gene expression. We have utilized a primary culture system of organoids composed of 10 to 50 cells. These organoids maintain cell–cell contacts similar to those found *in vivo* and can be cultured in serum-free hormonally defined conditions. In this system, the maintenance of differentiation depends on both ECM and hormonal signals. Since *in vivo* mammary cells differentiate on a basement membrane, we have determined the effects of a gel containing basement membrane components as well as a purified basement membrane component laminin on the production of a group of milk proteins. In order to characterize the levels of regulation we examined the effects of these basement membrane components on the steady state accumulation of mRNAs and proteins for α -, β -, and γ -casein and α -LA. We demonstrate that basement membrane gel and an isolated basement membrane component, laminin, induce milk protein gene expression. This modulation occurs at both the mRNA and protein levels. At the mRNA level, the effects of ECM components are greatest for α - and β -casein, less for α -LA, with no significant effect on γ -casein. These findings indicate that milk protein genes are not coordinately regulated by ECM components. The effects of ECM on α - and β -casein mRNAs may be due to increased transcription rates or decreased turnover of these messages.

Although matrix components have an effect on the steady state accumulation of mRNAs for milk protein genes, there are far greater effects at the protein level. For example, basement membrane gel induces α -casein accumulation greater than 160-fold relative to that on tissue culture plastic while the mRNA on this substratum is only fourfold greater than on tissue culture plastic. These results indicate that in our system a major effect of ECM components on mammary gene expression is at the translational and/or post-translational levels.

In order to determine whether differences in protein turnover or secretion could account for the increase in milk protein accumulation on ECM substrata we

performed pulse-chase experiments on cultures on laminin or tissue culture plastic. We chose a laminin substratum since pulse-chase and secretion studies are difficult to perform on gelled substrata due to the trapping of amino acids and secreted proteins within the gel. We demonstrated that on a laminin substratum after a 1-h pulse there was more than 27-fold more α 1-casein than on tissue culture plastic. By 1/2 h following the pulse, negligible amounts of α -casein were detected in the cell layer and trace amounts in the medium on tissue culture plastic. In contrast on laminin, α -casein turnover is reduced in the cell layer and it is more efficiently secreted into the medium. Laminin was also found to have an effect on transferrin secretion. On tissue culture plastic transferrin accumulated in the cell layer with little secretion into the medium. In contrast on laminin, the efficiency of transferrin secretion into the medium was greatly increased. Taken together, these results suggest that culturing these cells on laminin modulates milk protein intracellular turnover and secretion. This effect on protein secretion and accumulation is selective since total protein accumulation in the cell layer and medium as determined by TCA-precipitable counts is unaffected by substratum (data not shown). The elevated levels of α -casein on a laminin substratum after a brief pulse indicate that this substratum may influence translational events as well as protein turnover and secretion.

The relative increases in milk protein expression on ECM components represent induction of differentiated function rather than only dedifferentiation on tissue culture plastic. This induction occurs at both the mRNA and protein levels. The level of α -casein mRNA on plastic accumulated up to 25% of that expressed in the lactating gland with time in culture while on ECM substrata it accumulated up to 70% of the mRNA level found in the lactating mammary gland. Furthermore, the radioimmunoassay for α -LA indicates that the synthesis of this milk protein increases with time on the ECM components but remains essentially unchanged on tissue culture plastic. Therefore, as in the developing mammary gland [6], milk protein gene expression increases with time in this *in vitro* culture system in the presence of ECM components.

We have shown that mammary cells isolated from a hormonally primed virgin gland and maintained on ECM components are able to efficiently synthesize α -casein and α -LA. Although mRNAs for β - and γ -caseins are expressed in these cultures, we did not detect these milk proteins. This may reflect inefficient translation of these mRNAs and/or alterations in protein processing which prohibit detection by the polyclonal and monoclonal antibodies utilized.

Previous investigations into the role of ECM in mammary differentiation have primarily utilized floating gels of stromal collagen to induce mammary differentiation [1, 8-18]. In these culture systems the induction of differentiation requires contraction of the collagen gel. It has been suggested that cell-shape changes which accompany gel contraction may be responsible for this differentiation. Our studies demonstrate that basement membrane components can induce mammary differentiation in the absence of stromal collagen. Furthermore, these studies indicate that differentiation of rat mammary cells can occur on these basement membrane components in the absence of gel contraction. However, levels of

differentiation are higher on gelled matrices than on laminin-coated dishes. These observations suggest that both the composition of the ECM and its geometry may play a role in the induction of differentiated function.

A number of investigators in other cellular systems have shown that complex extracellular matrices as well as purified basement membrane components play a role in modulating differentiated function [43–48]. In the liver, one of the best studied systems other than the mammary gland, Reid and co-workers have shown that isolated basement membrane components induce liver-specific mRNAs primarily at a post-transcriptional level by altering the stability of liver-specific mRNAs [43]. Additionally, proteoglycans have been shown to induce the expression of liver-specific genes and reduce the expression of common genes to levels comparable to *in vivo*. Proteoglycans appear to modulate gene expression by both transcriptional and post-transcriptional mechanisms [43, 44]. Moreover, in the liver system, proteoglycans alter the expression of gap junctional proteins, promote cellular coupling, and affect cell shape in culture [44, 46]. These studies in addition to our own suggest that ECM components influence multiple aspects of cell behavior including tissue-specific gene expression, cytoskeletal protein gene expression, cell–cell communication, and cell shape. Moreover, these studies confirm that ECM components affect gene expression at multiple regulatory levels.

How can a single ECM component such as laminin effect the expression of milk protein genes at multiple levels? Although the molecular mechanisms involved remain to be determined, one possibility is that these events are mediated by the cytoskeleton. It has been proposed that the cytoskeleton may be involved in gene expression in several different ways. Actin and intermediate filaments have been shown to extend from the cytoplasm into the nucleus where they form part of the nuclear matrix [49, 50]. There is evidence that the nuclear matrix may play a role in tissue-specific gene expression [51, 52]. Other studies have indicated that mRNAs and polyribosomes are associated with the cytoskeleton [53–55]. Thus, mRNA stability or translational efficiency may be altered by association of specific mRNAs with the cytoskeleton. Furthermore, there is evidence for a role of the cytoskeleton in protein secretion [56]. We have recently demonstrated that the treatment of primary rat mammary cultures with cytochalasin D to disrupt the actin cytoskeleton selectively inhibits laminin-induced α -LA synthesis without having a major effect on general protein synthesis [57]. These experiments suggest the effects of laminin on mammary differentiation require an intact actin cytoskeleton.

Our group and others have demonstrated that cells interact with the basement membrane component laminin via a specific 68-kDa cell surface receptor [58]. Furthermore, we demonstrated that this laminin receptor is able to interact with the cellular cytoskeleton [59, 60]. These findings raise the possibility that ECM components might exert their major effects on mammary differentiation by altering the cellular cytoskeleton through these receptors. The cytoskeleton may in turn determine cell shape and influence gene expression at multiple levels. The ability to induce mammary differentiation with purified ECM components should

allow the study of the interaction of these components, their receptors, and the cytoskeleton in the control of mammary differentiation.

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