New Mammary Epithelial and Fibroblastic Cell Clones in Coculture Form Structures Competent to Differentiate Functionally

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Abstract. We have established and characterized a spontaneously immortalized, nontumorigenic mouse mammary cell line, designated IM-2. IM-2 cells synthesize large amounts of the milk protein β-casein upon addition of lactogenic hormones. The induction of β-casein occurs rapidly and does not require any exogenous extracellular matrix components. The IM-2 cell line is morphologically heterogeneous and could be separated into cell clones with epithelial and fibroblastic characteristics. In monoculture, none of the epithelial clones could be induced to synthesize caseins. Coculture of epithelial and fibroblastic clones, however, rendered the epithelial cells competent to differentiate functionally; the addition of lactogenic hormones to these cocultures resulted in the synthesis of β-casein in amounts comparable to that seen with the original IM-2 line.

Using this unique cell system, we have investigated the interrelationships between different steps in differentiation leading to hormone-induced casein production. Independent of hormones, epithelial–fibroblastic cell contacts led to the formation of characteristic structures showing the deposition of laminin. We found that the epithelial cells located in these structures also exhibited significantly increased levels of cytokeratin intermediate filament polypeptides. Double immunofluorescence revealed that the cells inducible by hormones to synthesize casein, colocalized exactly with the areas of laminin deposition and with the cells showing greatly intensified cytokeratin expression. These results suggest that hormone-independent differentiation events take place in response to intercellular epithelial–mesenchymal contacts. These events in turn bring about a state of competence for functional differentiation after lactogenic hormonal stimulation.

THE epithelial component of the mammary gland is an exceptional tissue in that its proliferation and differentiation can be induced in the fully developed adult according to its hormonal status (Topper and Freeman, 1980). The study of growth and differentiation in mouse mammary epithelium presents an interesting opportunity for understanding tissue-specific gene expression and its relationship to tissue organization and hormonal influences. Much work has been directed toward studying primary cell preparations enriched in the epithelial component of the mammary gland. These studies had to be confined to short term primary cultures, partly because of the difficulty of maintaining the growth potential of these cells and partly because of complications due to overgrowth of nonepithelial cell types. There is therefore a need for cell lines of mammary epithelial origin. To date, only a few nonneoplastic mouse mammary cell lines of epithelial origin have been es-

tablished (Anderson et al., 1979; Howard et al., 1983; Danielson et al., 1984). Of these, only the COMMA-1D line could be induced to synthesize appreciable amounts of casein in vitro (Danielson et al., 1984). COMMA-1D apparently contains a pluripotent mammary stem cell-like population which, after subcloning, gives rise to phenotypically heterogenous cell clones (Medina et al., 1986; Ball et al., 1988; Campbell et al., 1988). Unfortunately, it has been shown that COMMA-1D cells exhibit increasing oncogenic potential after prolonged passage (Medina et al., 1986).

The organization and function of mammary epithelium are markedly influenced by the substrata upon which these cells are cultured. It has been demonstrated that collagen gels (Emerman and Pitelka, 1977; Lee et al., 1985), mammary gland "biomatrix" (Wicha et al., 1982), and basal lamina components extracted from Engelbreth-Holm-Swarm tumors (Li et al., 1987) can foster mammary gland differentiation. However, these artificially reconstituted substrates do not account for the complexity of the epithelial–fibroblastic cell interactions. Levine and Stockdale (1985) and Wiens et al. (1987) demonstrated that primary cell preparations enriched in the epithelial component of the murine mammary gland exhibited proliferation and hormone-dependent differentia-

tion when cocultured with adipocytes or preadipocytes. It has also been demonstrated that the fibroblastic mesenchyme located in close apposition to the respective epithelium also determines epithelial cell differentiation (Kratochwil and Schwartz, 1976; Sakakura, 1983). Thus, it is evident that both mesenchymes influence and contribute to mammary epithelial differentiation.

We report here the establishment and characterization of a nontumorigenic mouse mammary cell line, designated IM-2. IM-2 cells can be rapidly induced to synthesize large amounts of β-casein on conventional cell culture plastic, independent of the addition of any exogenous extracellular matrix components. Single-cell cloning of IM-2 and the subsequent two-dimensional polyacrylamide gel analysis of intermediate filament polypeptides extracted from the different cell clones, revealed that IM-2 was a heterogeneous cell population consisting of epithelial and fibroblast-like cells. Both epithelial and fibroblastic cell clones were found to be unresponsive to lactogenic hormones when grown in monoculture. Coculture of clonal mammary epithelial and fibroblastic cells, however, resulted in the development of characteristic structures in which there was a correlation between the deposition of laminin, the increased expression of cytokeratins, and the competence to respond to lactogenic hormones by synthesizing and secreting caseins.

Materials and Methods

Establishment and Culture of Mammary Cell Lines

The fourth mammary glands of mid-pregnant BALB/c mice were finely minced and dispersed by collagenase treatment as described (Voyles and McGrath, 1976). Isolated cell aggregates enriched in the epithelial component were plated in growth medium consisting of DME (Gibco Laboratories, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, insulin (5 μg/ml), EGF (10 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Primary cell cultures were incubated at 37°C in a water-saturated atmosphere containing 5% CO_2 in air. Overgrowing fibroblasts were removed by differential trypsinization. The growth of epithelial-like cell colonies became apparent after 12 wk of culture without passage. Cultures reached confluence 5 wk later and were thereafter split 1:2 at 2-wk intervals. After surviving a growth crisis, cells were tested for their ability to synthesize caseins in response to lactogenic hormones, a property exhibited by only one (IM-2) of three isolates obtained. At present, IM-2 cells are subcultured 1:3 weekly in a low oxygen atmosphere of 5% O_2, 6% CO_2, and 89% N_2 to minimize the generation of mutagenic oxygen radicals.

Casein Induction

Cells were grown on plastic Petri dishes in growth medium until they reached confluence. Hormone induction medium was then added, consisting of DME supplemented with 5% FCS, insulin (5 μg/ml), hydrocortisone (1 μg/ml), and ovine prolactin (5 μg/ml). The cultures were incubated at 37°C for an additional 3 d during which time the medium was changed once.

Preparation of Rabbit Anti-β-Casein Antiserum

Milk was obtained from lactating BALB/c mice by use of a suction device 15–30 min after intraperitoneal injection of 2.5 U of oxytocin. Caseins were recovered by acid precipitation (pH 4.6) of skim milk according to the method of Enami and Nandi (1977). The 29-kD β-casein–specific polypeptide was separated from other caseins by SDS-PAGE. Gels were stained by Coomassie Blue and the band corresponding to the 29-kD β-casein protein was cut out from the gel. Gel slices were minced finely and the 29-kD protein was eluted overnight at room temperature in PBS. The eluate was concentrated by lyophilization and subsequently dialyzed against PBS. A New Zealand female rabbit was injected intradermally with 300 μg of β-casein in complete Freund's adjuvant. After two booster immunizations (3 and 6

wk later, respectively) with 300 μg of the 29-kD protein in incomplete Freund's adjuvant, the rabbit was bled and serum isolated.

Western Blot Analysis

Cells were washed twice with PBS at the end of a hormone induction period. Extracts were prepared by scraping cells into RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 40 mM NaF, 1% Triton X-100, 1% sodium-deoxycholate, and 0.1% SDS), followed by homogenization with a glass homogenizer (Dounce, Vineland, NJ). Debris were pelleted and the supernatant was collected and diluted in twofold concentrated SDS sample buffer (Laemmli et al., 1970). Proteins in culture supernatants were precipitated by adding 5 vol of acetone for 1 h at −20°C. After centrifugation the dried pellet was dissolved in SDS sample buffer. The protein concentration of the samples was determined by the method of Schaffner and Weissman (1973). SDS-PAGE was performed as described by Laemmli (1970) in 11% polyacrylamide gels. Caseins were transferred from gels to nitrocellulose at 0.8 mA/cm^{2} for 90 min using a semi-dry electroblotter (Kyhse-Andersen, 1984). After blocking nonspecific binding with 0.5% gelatine in PBS, the nitrocellulose was exposed to antibodies diluted 1:1,000 and reacted with ^{125}I-labeled protein A. The labeled antigen–antibody–protein A complex bound to nitrocellulose was detected by autoradiography with x-ray film (XAR-5; Eastman Kodak Co., Rochester NY).

Northern Blot Analysis

Total RNA was prepared using the guadinium thiocyanate extraction method followed by a CsCl gradient centrifugation according to Chirgwin et al. (1979), and poly A$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). RNA was denatured with glyoxal and separated on a 1% agarose gel in phosphate buffer before blotting onto nitrocellulose in 20× SSC (Maniatis et al., 1982). Hybridization was carried out at 42°C using a 0.5-kb ^{32}P-dCTP nick-translated mouse β-casein c-DNA probe (Hennighausen and Sippel, 1982) in a buffer containing 50% formamide, 4× SSC, 5× Denhardt's solution, 0.1% sodium pyrophosphate, 0.25 mg/ml yeast tRNA, 0.2% SDS, and 30 μg/ml salmon sperm DNA. The filters were washed twice at 42°C for 30 min in 2× SSC, 0.2% SDS followed by 0.5× SSC, 0.2% SDS before autoradiography.

Analysis of Cytoskeletal Extracts by Two-Dimensional Gel Electrophoresis

Cell extracts enriched in intermediate filament polypeptides were prepared from cell cultures using the procedure described by Franke et al. (1981). Briefly, the cell layer was treated for 3–5 min at room temperature with TN buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 1% Triton X-100). Thereafter, cells were incubated for 30 min at room temperature in high salt buffer (TN buffer containing 1.5 M KCl and 0.5% Triton X-100). The insoluble residual cell proteins were scraped off and collected by 20 min centrifugation at 3,500 g. The pellets obtained were washed twice in PBS and the pellets were processed for gel electrophoresis. One-dimensional SDS-PAGE was performed according to Laemmli (1970) using a 4.2% polyacrylamide stacking gel (1.5 cm) and a 14% running gel (12 cm) at constant voltage (70 V) overnight at room temperature. Two-dimensional PAGE using NEPHGE in the first dimension was performed according to O'Farrell (1975, 1977). Extract pellets were solubilized for 2 h at room temperature in 9.2 M urea containing 2% ampholytes (pH 3.5–11.0; Serva Fine Biochemicals Inc., Garden City Park, NY) and 5 mM DTT, and was subjected to electrophoresis for 4–5 h (1,600–2,000 V-hours). First-dimension gels were equilibrated in 2% SDS, 60 mM Tris-HCl, 5% β-mercaptoethanol, 10% glycerol, pH 7.1 for 30 min and then placed on 11% slab gels for electrophoresis overnight at room temperature. Molecular mass standards included were BSA (68 kD) and phosphoglycerate kinase (43 kD). Gels were subsequently stained in Coomassie Brilliant Blue and stored in a sealed plastic bag at 4°C.

Immunocytochemistry

Cells cultivated on plastic culture dishes were washed twice with PBS, fixed, and permeabilized for 3 min using a 1:1 mixture of acetone and methanol at −20°C, and then air dried. The first antibody was applied for 90 min at room temperature in a humid atmosphere, followed by five washes in PBS, and application of 1:50 dilution of fluorchrome-conjugated antirabbit IgG or goat anti-mouse IgG (Cappel Laboratories, Malverne, PA). After staining, the cells were mounted in Mowiol (Hoechst) containing 50
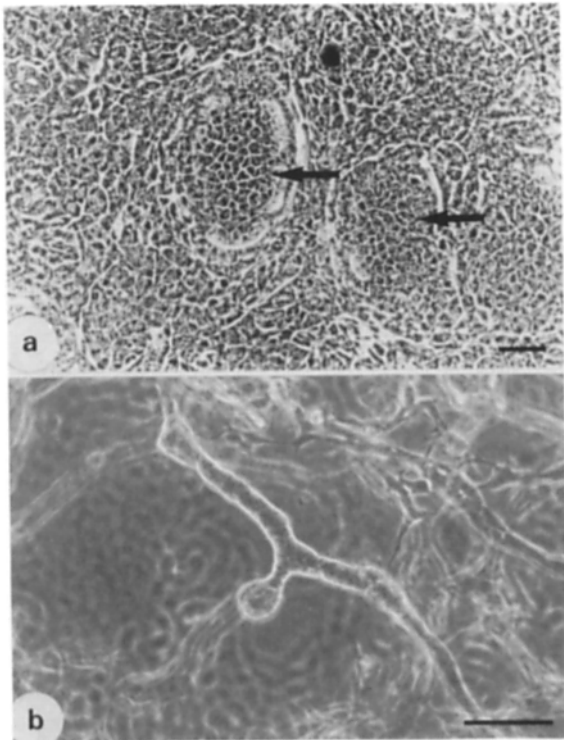
Figure 1. Phase-contrast micrographs of IM-2 cells. (*a*) IM-2 cells plated on cell culture plastic show islands of cells with an epithelial morphology (*arrows*) which are surrounded by a layer of fibroblast-like cells. (*b*) Duct-like outgrowth of IM-2 cells embedded within a collagen type I gel. Bars, 100 μm.

mg/ml 1,4-diazobicyclo [2,2,2] octane as an antiquenching agent, and viewed using a Leitz fluorescence microscope with epi-illumination.

Antibodies

The mouse monoclonal antibody LE 61 recognizing human cytokeratin 18 and related cytokeratins in other species, was generously provided by Dr.

Results

IM-2 Cells Display Properties Related to Those of Functional Mammary Epithelium

The IM-2 cell line was derived from mammary tissue isolated from the fourth mammary glands of mid-pregnant BALB/c mice. Morphological inspection of the spontaneously immortalized line revealed that it apparently consisted of epithelial-like cells growing in islands surrounded by fibroblast-like cells (Fig. 1 *a*). When embedded in collagen type I gels, IM-2 cells formed three-dimensional duct-like outgrowths (Fig. 1 *b*) similar to those reported for primary mammary epithelial cells (Richards et al., 1982). No tumors resulted after subcutaneous injection of 10^6 IM-2 cells into each of five BALB/c and five nude mice during an observation period of three months.

To compare the IM-2 population with the primary cell preparation from which they were derived, and to gain information about the lineage of the cells contained in IM-2, intermediate filament polypeptides were extracted from both IM-2 and freshly isolated mouse mammary cells, enriched for the epithelial component. Two-dimensional PAGE of detergent-insoluble proteins revealed patterns of intermedi-

← NEPHGE
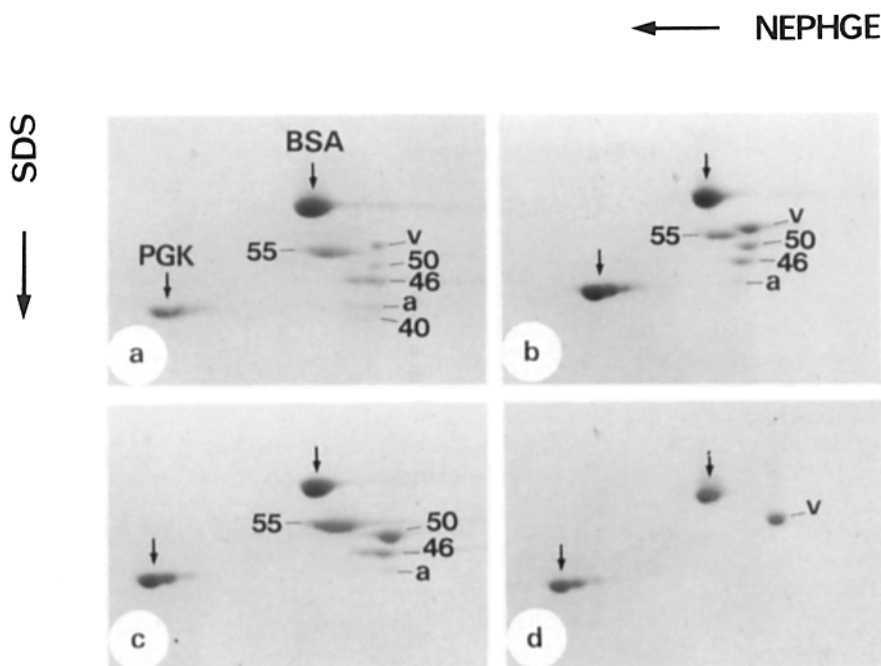
Figure 2. Two-dimensional PAGE of the detergent-insoluble, cytoskeletal protein fraction isolated from (*a*) primary cultures of mouse mammary organoids, (*b*) IM-2 cells, (*c*) clonal Cl 31E cells, and (*d*) clonal Cl 30 F cells. The numbers indicate the molecular masses of cytokeratins in kilodaltons. *v*, vimentin (58 kD); *a*, actin (43 kD). Phosphoglycerate kinase (*PGK*, 43 kD) and BSA (*BSA*, 68 kD) are marker proteins.

a

Mg   0   3   8   24 hours

18 S –

ß-cas

b

milk   0   1   2   3   4   5   6   7   8 days

α1
α2

ß
γ
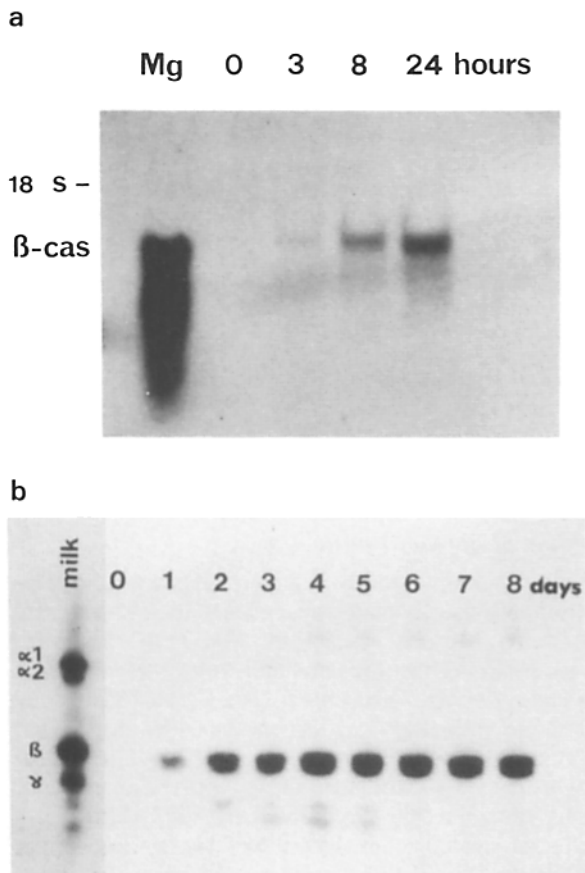
Figure 3. Casein production in lactogenic hormone-treated IM-2 cells. (a) Northern blot analysis of β-casein mRNA: poly A–selected mRNAs obtained from cells stimulated with lactogenic hormones for increasing time intervals were resolved on agarose gels and blotted onto cellulose nitrate. Detection was with a β-casein-specific nick-translated probe. The locations of the 18-S ribosomal RNA and the 1.5-kb β-casein mRNA are indicated. *Mg,* mRNA prepared from the lactating mouse mammary gland. (b) Immunoblot analysis of β-casein protein: parallel plates of IM-2 cells treated with lactogenic hormones were lysed at daily intervals with RIPA buffer and equal protein amounts were resolved by SDS-PAGE followed by electroblotting onto cellulose nitrate. Intracellular caseins were detected using rabbit antiserum to mouse milk caseins, followed by ^{125}I-protein A. Caseins purified from mouse milk are included for comparison: α1(43 kD), α2(42 kD), β(31 kD), and γ(26 kD).

ate filament polypeptides that were almost identical in IM-2 and in primary mammary epithelial cell preparations (Fig. 2, *a* and *b*). Four major cytokeratins of 55, 50, 46, and 40 kD, as well as vimentin (58 kD), were found in primary preparations of mammary epithelial cells (Fig. 2 *a*). Intermediate filament proteins of identical molecular masses and isoelectric properties were detected by Asch and Asch (1985) in primary mouse mammary epithelial tissues. In IM-2 cells, three cytokeratin proteins of 55, 50, and 46 kD as well as vimentin were detected, while the 40-kD cytokeratin species was not found in IM-2 extracts (Fig. 2 *b*). These results show that mammary epithelial cells are contained in the IM-2 population.

To investigate the induction of milk-protein synthesis, con-

fluent IM-2 cells plated on tissue culture plastic dishes were exposed to hydrocortisone, insulin, and prolactin (HIP) for 0, 3, 8, and 24 h. The analysis of poly A$^+$ mRNA revealed that β-casein-specific mRNA (1.5 kb) was detectable as early as 3 h after hormonal induction (Fig. 3 *a*). Compared with the amount of β-casein mRNA detected 3 h after hormone treatment, the 8-h sample showed a 10-fold increase and that analyzed after 24 h, a 30-fold increase (Fig. 3 *a*).
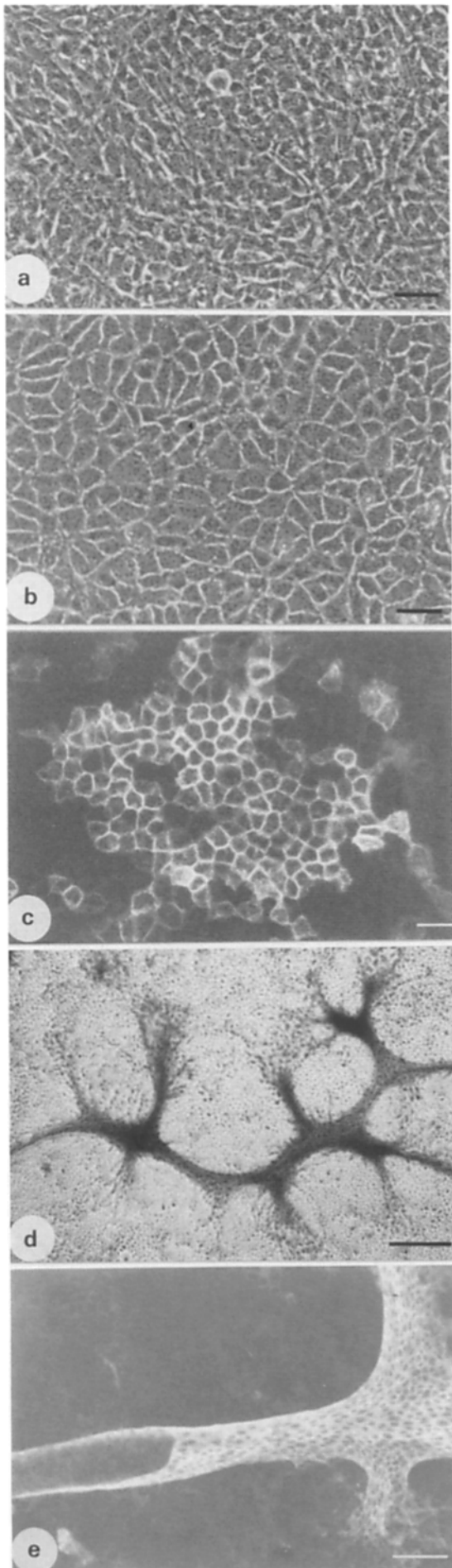
Intracellular β-casein could be detected by immunoblot analysis within 24 h after hormone treatment (Fig. 3 *b*). Maximal expression was reached after 3 d of induction and stayed constant for the remaining 5-d period of investigation (Fig. 3 *b*). Cytoplasmic β-casein had an apparent molecular mass of 29 kD as compared to 32 kD for secreted milk casein (Fig. 3 *b*). The difference in molecular mass between intracellular β-casein and secreted β-casein has been described previously (Durban et al., 1985) and presumably reflects differences in posttranslational modifications; e.g., phosphorylation of casein polypeptides. The molecular mass of β-casein secreted into the culture medium corresponded to that of β-casein found in mouse milk (see below).

IM-2 Comprises Epithelial and Fibroblast-like Cell Types

Since IM-2 seemed to be a morphologically heterogeneous cell population, we isolated and characterized the different cell types included in IM-2 by single cell cloning. Cloning was performed taking advantage of the neomycin resistance gene contained in a retroviral vector. Infection of 10^6 IM-2 cells at a multiplicity of infection of <0.01 with the recombinant retrovirus pSVX (Cepko et al., 1984) generated 150–200 G-418–resistant colonies of various morphologies. 10 such colonies were selected and subjected to a second cycle of cloning by limiting dilution, after which the intermediate filament polypeptides of each of the clones were analyzed.

Two-dimensional PAGE of detergent-insoluble proteins revealed that the cell clones comprised two groups, each with a characteristic pattern (Fig. 2, *c* and *d*). Two clones of the first group exhibited an epithelial morphology and expressed three major cytokeratins of 55, 50, and 46 kD (Fig. 2 *c*). The clones of the second group expressed one intermediate filament protein of 58 kD corresponding to vimentin (Fig. 2 *d*). When extracts of the two groups were mixed, the intermediatae filament polypeptide pattern typical for the IM-2 population was reconstituted (data not shown).

A representative clone from each group (Cl 30F and Cl 31E) was chosen for further analysis. The morphology of fibroblast-like Cl 30F cells ranged from elongated to polygonal, depending on cell density (Fig. 4 *a*). Cl 31E cells exhibited a typical epithelial morphology (Fig. 4 *b*) and formed monolayers of cells in close contact, tightly adherent to the plastic substrate. Confluent cultures of Cl 31E cells developed hemicysts or domes which are believed to be due to unidirectional fluid secretion of polarized epithelial cells when cultured on a plastic substrate (Lever, 1979; Sugahara et al., 1984). Using the monoclonal antibody 281-2 (Jalkanen et al., 1985), which recognizes the core protein of cell surface heparan-rich proteoglycans (HSPG), Cl 31E cells exhibited the basolateral staining (Fig. 4 *c*) typical of polarized epithelial monolayers (Repraeger et al., 1986). In monocul-

ture, Cl 31E cells stained weakly with the mouse monoclonal antibody LE 61 which recognizes cytokeratins related to human cytokeratin 18 (Lane, 1982). After 8 mo in continuous culture (72 passages), Cl 31E cells were still not tumorigenic as determined by subcutaneous injection of 10^6 cells in each of nine nude mice. During 3 mo of observation no palpable tumors were detected.

Structural and Functional Differentiation Is Mediated through Epithelial-Fibroblastic Cell Contact

Structural and functional differentiation failed to occur in monocultures of Cl 31E and Cl 30F cells. To reconstitute the differentiated properties of the heterogeneous IM-2 cell line, we cocultured epithelial Cl 31E cells either with fibroblast-like Cl 30F cells or with NIH 3T3 fibroblasts. Two different coculture protocols were employed. In the first, a mixed cell suspension was plated onto petri dishes. This resulted in the formation of a pure epithelial monolayer, on top of which fibroblastic branching cords developed (Fig. 4 *d*). These fibroblastic bundles served as a new substrate which supported the formation of a regular epithelial cell layer completely or partially surrounding the fibroblastic core, as demonstrated by cytokeratin staining (Fig. 4 *e*). These structures can be regarded as inverted or inside-out mammary "ducts". Double immunofluorescence with anti-fibronectin (mesenchyme-specific; Fig. 5 *a*) and anti-cytokeratin (epithelium-specific; Fig. 5 *b*) immune reagents demonstrate the organization of the cords (Fig. 5, *a*, *b*, and *c*). Epithelial cells in close contact with the fibroblastic bundle exhibited a greatly increased cytokeratin staining when compared with Cl 31E cells directly adherent to cell culture plastic (Fig. 5 *b*). Anti-vimentin antibody (mesenchyme-specific; Fig. 5 *d*) was used in conjunction with an anti-β-casein antibody (Fig. 5 *e*) in double immunofluorescence on cocultures to illustrate the localization of hormonally responsive cells (Fig. 5, *d*, *e*, and *f*). Strikingly, we found that it was almost exclusively the epithelial cells in close apposition to the central fibroblastic bundle which synthesized casein after lactogenic hormone stimulation. The lower left and upper right quadrants in Fig. 5 *e* show regions of pure epithelial monolayers which are seen to be almost devoid of casein-specific staining.

Immunoblot analysis confirmed the results obtained with immunofluorescence. Neither Cl 31E nor Cl 30F cells in monoculture synthesized caseins when stimulated with lactogenic hormones. Coculturing a mixture of the two clones, however, rendered the epithelial cells competent to respond to lactogenic hormones (Fig. 6).

Casein synthesis in these cocultures was markedly influenced by the ratio of the two cell clones. Plating Cl 31E and

Figure 4. (*a* and *b*) Phase contrast micrographs illustrating the appearance of fibroblastic Cl 30F cells (*a*) and epithelial Cl 31E cells (*b*) in pure culture. (*c*) The baso-lateral localization of a cell surface proteoglycan on Cl 31E cells detected by monoclonal antibody 281-2. (*d*) Phase-contrast micrograph of three-dimensional branching cords that develop in cocultures of Cl 31E and Cl 30F cells initiated as suspensions of equal numbers of the two cell types. (*e*) The composition of the cords is demonstrated using the cytokeratin-specific antibody LE 61 in indirect immunofluorescence. Bars, (*a* and *b*) 50 μm; (*c*) 20 μm; (*d*) 200 μm; (*e*) 100 μm.
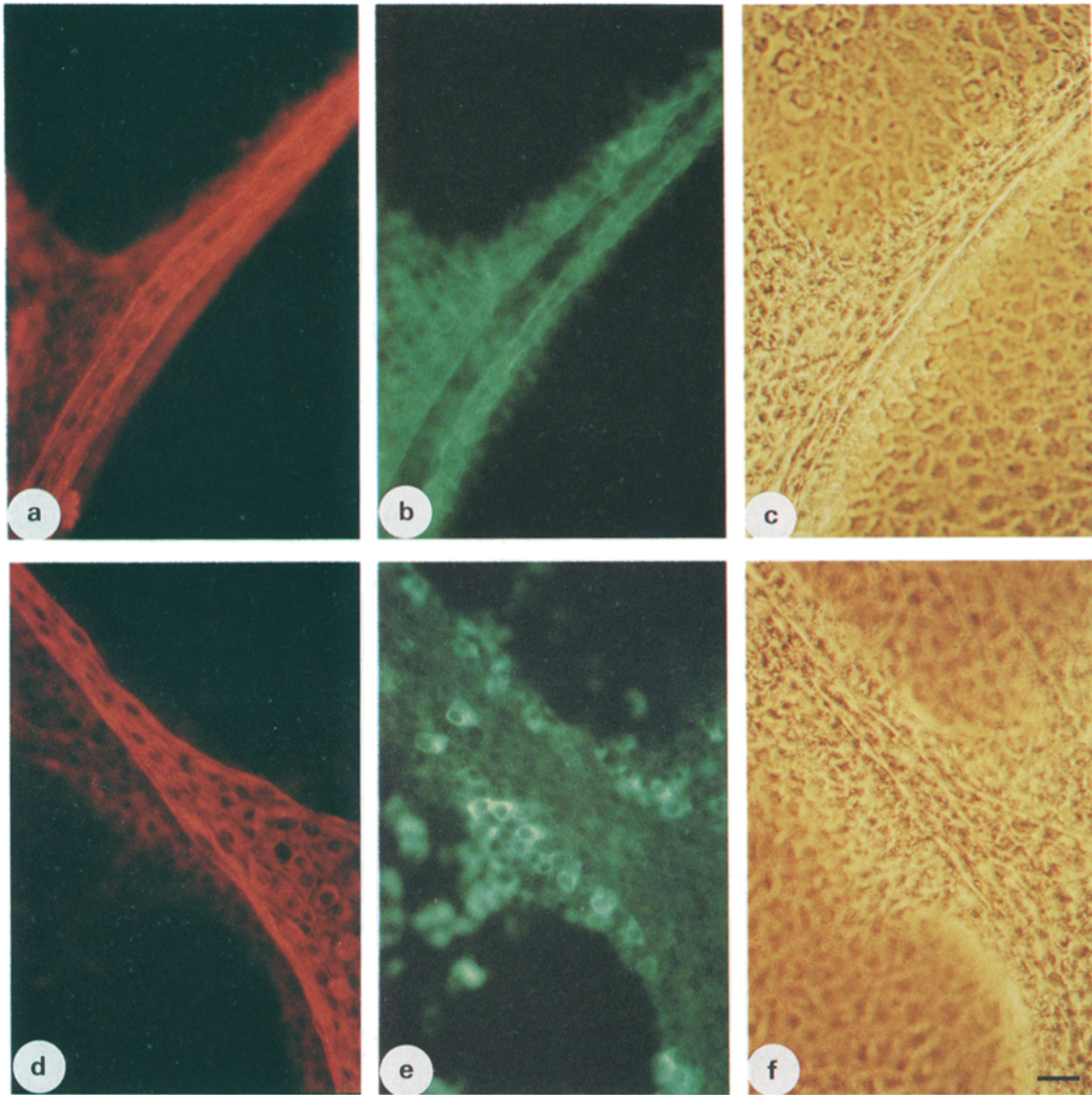
Figure 5. Double immunofluorescence demonstrates the composition of three-dimensional cords resulting from 1:1 coculture of Cl 31E cells and NIH 3T3 fibroblasts. (*a*) The central bundle of fibroblasts is visualized using an anti–fibronectin antiserum. (*b*) Cl 31E cells partially surrounding the fibroblastic bundle are delineated using cytokeratin-specific antibodies (LE 61). (*c*) The same field as in *a* and *b*, seen in phase contrast. (*d*) Cocultures stained with vimentin-specific antibodies. (*e*) The same field as in *d* stained with β-casein antiserum after hormonal stimulation. (*f*) The same field photographed in phase contrast. Bar, 20 μm.

Cl 30F cells in a ratio of 1:1 resulted in the formation of three-dimensional cords and allowed maximal β-casein production (Fig. 6 *A*, lane *f*). Similar results were obtained when NIH-3T3 fibroblasts (Fig. 6 *a*, lane *h*) or mouse embryo fibroblasts (data not shown) were substituted for Cl 30F cells. Thus, fibroblastic cells from other organs of the mouse were able to render mammary epithelial cells competent to differentiate functionally. Under the same conditions, the secretion of β-casein was also observed (Fig. 6 *b*, lane *c*). The difference in molecular masses between intracellular and secreted β-casein has previously been described by Durban et al. (1985).

In a second coculture procedure, a suspension of epithelial cells was plated on a preformed confluent monolayer of fibroblastic cells. In this case, clusters of epithelial cells developed on top of the fibroblastic monolayer in such a way that every epithelial cell was in apposition to fibroblastic cells (Fig. 7). Cytokeratin-specific immunofluorescence (Fig. 7 *a*) and phase-contrast microscopy (Fig. 7 *c*) document the organization of these double-layered structures. With this second coculture technique, almost 100% of the epithelial cells responded to lactogenic hormones by synthesizing β-casein (Fig. 7 *b*).

To investigate the nature of interaction between the epithelial and the fibroblast-like cell types leading to competence for hormone response, medium conditioned by Cl 30F cells was added to cultures of Cl 31E. Subsequent addition of lactogenic hormones did not result in the synthesis of β-casein

a

b
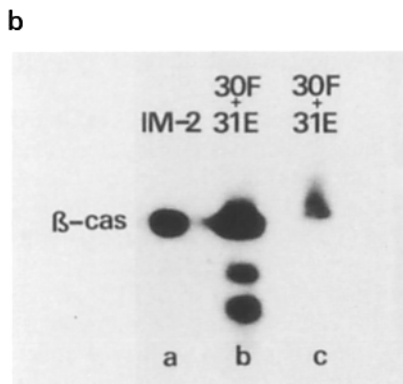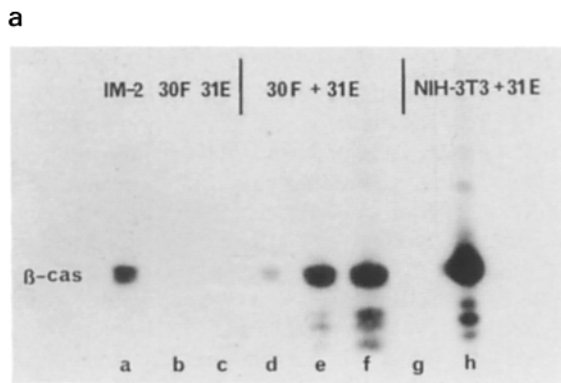
Figure 6. (*a*) Immunoblot analysis using a rabbit anti–β-casein antiserum of caseins extracted from pure cultures of fibroblastic Cl 30F cells (lane *b*), mammary epithelial Cl 31E cells (lane *c*), and mixtures of the two clones (lanes *d*, *e*, and *f*) at the ratios Cl 30F/Cl 31E 1:10 (lane *d*), 1:2 (lane *e*), and 1:1 (lane *f*). The coculture of Cl 31E with NIH-3T3 fibroblasts at a ratio of 1:1 also resulted in an efficient casein synthesis (lane *h*). Caseins extracted from IM-2 cells serve as a reference (lane *a*). Lane *g* is vacant. (*b*) Immunoblot analysis of caseins synthesized by Cl 31E cells arranged in three-dimensional cords (1:1 coculture). Lane *a*, β-casein standard extracted from IM-2 cells. Lane *b*, intracellular caseins extracted from 1:1 mixtures of Cl 31E and Cl 30F cells. Lane *c*, secreted β-casein isolated from culture medium of 1:1 cocultures of Cl 31E and Cl 30F cells.

(data not shown). In contrast, plating 2 × 10^6 epithelial Cl 31E cells on a confluent monolayer of Cl 30F cells that was either unmanipulated (Fig. 8 *a*, lane *b*), lethally irradiated (Fig. 8 *a*, lane *c*), or fixed by 4% formaldehyde (Fig. 8 *a*, lane *d*) resulted in the efficient synthesis of β-casein upon hormone induction. This result suggests that components deposited by fibroblastic cells, presumably extracellular matrix molecules, are involved in mediating functional differentiation of mammary epithelial Cl 31E cells. In support of this conclusion, we found that type I collagen gels could substitute for fibroblastic cells in the lactogenic hormone induction of casein (Fig. 8 *b*, lane *b*).

Epithelial–Mesenchymal Cell Interactions Elicit Increased Laminin Deposition

The results described above suggest that components located on the surface of fibroblastic cells are required for the establishment of differentiated functions in mammary epithelial Cl 31E cells. A possible explanation for this finding is that these components are involved in the deposition of a functional basal lamina. If this were the case, Cl 31E cells cultured on top of a confluent monolayer of fibroblastic cells should more efficiently deposit laminin than the same cells grown on cell culture plastic. To test this hypothesis, we examined the production of the basal lamina component laminin in monocultures and cocultures of Cl 31E and Cl 30F cells.

Culture medium was analyzed for secreted laminin by SDS-PAGE and immunoblotting using an antiserum directed against mouse laminin. Murine laminin is composed of three polypeptide subunits: an A (440 kD), a B1 (225 kD), and a B2 chain (205 kD), linked by disulfide bonds in a cruciform structure (Timpl et al., 1979; Engel et al., 1981; Sasaki et al., 1987). The two polypeptides B1 and B2 could be detected in culture media of Cl 30F monocultures, whereas only the B1 polypeptide was secreted by Cl 31E (Fig. 9 *a*, lanes *c* and *d*, respectively). The A chain could not be detected in the culture media. This 400-kD subunit has been reported either not to be made at all or to undergo rapid degradation in a number of cell lines in vitro (Oliver et al.,
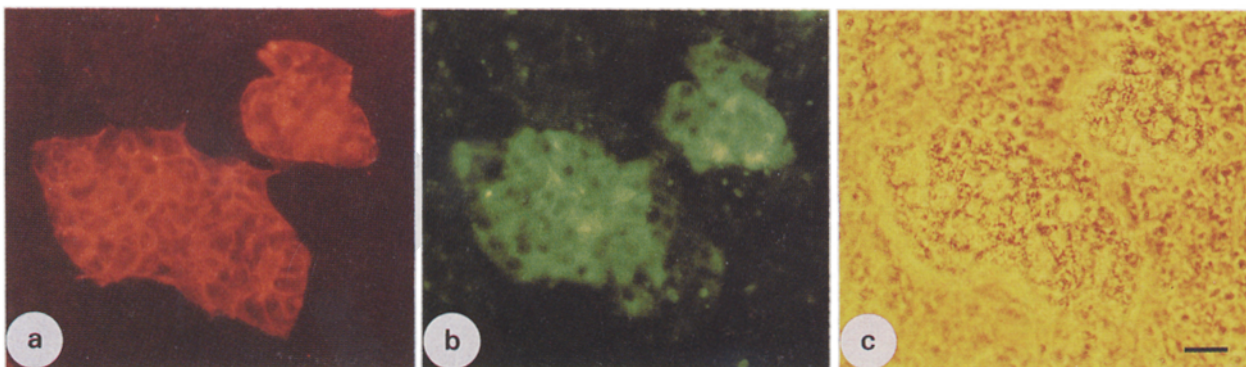
Figure 7. Clusters of mammary epithelial Cl 31E cells on top of a preformed monolayer of NIH-3T3 fibroblasts were double-stained with the cytokeratin-specific antibody LE 61 (*a*) and with an antiserum directed against β-casein (*b*). The respective phase-contrast micrograph is shown in *c*. Note that >90% of the epithelial cells synthesize casein under these culture conditions. Bar, 20 μm.

a

b
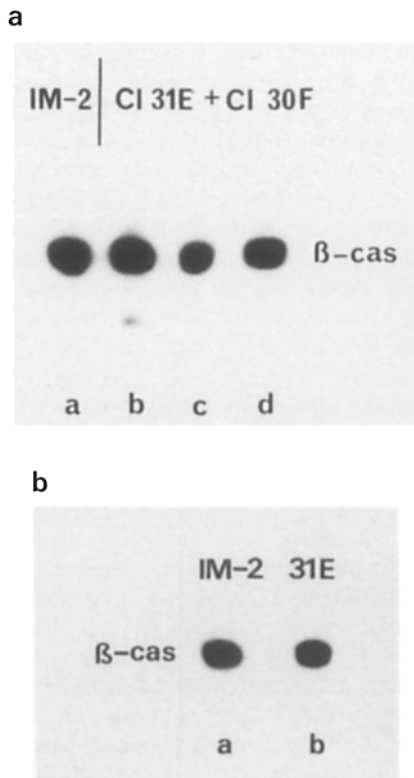
Figure 8. (*a*) Immunoblot showing intracellular β-casein detected by means of a rabbit antiserum. An extract from IM-2 cells serves as a standard (lane *a*). Cytoplasmic extract of Cl 31E cells growing on three differently treated monolayers of fibroblastic Cl 30F cells: lane *b*, intact and living; lane *c*, lethally irradiated; lane *d*, formaldehyde-fixed. (*b*) Immunoblot illustrating β-casein production by Cl 31E cells growing in pure culture on a collagen type I gel (lane *b*). Lane *a*, β-casein standard.

1983; Cornbrooks et al., 1983; Lander et al. 1985). Recently, Klein et al. (1988) have shown that A chain synthesis is transiently associated with specific differentiation events.

Mixing constant numbers of Cl 31E with increasing numbers of Cl 30F resulted in a simple additive increase of B1 and B2 laminin subunits detectable in the medium (Fig. 9 *a*, lanes *e*, *f*, and *g*).

The deposition of laminin was investigated by immunocytochemical methods. Fibroblastic Cl 30F cells in pure culture exhibited a diffuse cytoplasmic immunofluorescence, but lacked the discrete fibrils of deposited laminin sporadically seen on the surfaces of epithelial Cl 31E cells (Fig. 9, *b* and *c*). Thus, although both cell types synthesize and secrete laminin subunits, it is only the epithelial clone that seems to possess the potency to anchor laminin on its surface. Coculture of Cl 31E on a preformed monolayer of Cl 30 F cells resulted in a large increase in amounts of deposited laminin in areas where epithelial cell clusters were in intimate contact with the fibroblastic layer. This is demonstrated by double immunofluorescence using anti–cytokeratin and anti–laminin immune reagents in Fig. 9, *d* and *e*, respectively.

The Coculture of Cl 31E Cells with Fibroblastic Cells Results in Cytokeratin Accumulation

Immunofluorescence data presented above (Figs. 4 *e* and 5 *b*) show that Cl 31E grown in close contact with fibroblastic cells exhibit a greatly increased cytokeratin staining as compared to their counterparts adhering to culture plastic. For a quantitative evaluation of this phenomenon, the detergent-insoluble fraction of total cell extracts was resolved by one-dimensional SDS-PAGE and subsequently stained by Coomassie Brilliant Blue. Extracts were prepared from monocultures of NIH 3T3 fibroblasts and Cl 31E cells as well as from cocultures initiated by 1:1 mixtures. The amounts of proteins loaded onto the gel were normalized with respect to the total detergent-soluble protein that could be extracted from each sample. The extracts of NIH 3T3 cells contained vimentin, but no cytokeratin intermediate filaments (Fig. 10 *a*, lane *b*).

A confluent monolayer of epithelial Cl 31E cells exhibited moderate levels of the three cytokeratins already described in Fig. 2, *b* and *c*, but no vimentin (Fig. 10 *a*, lane *c*). Coculture, however, resulted in an ∼6–10-fold increase in the levels of both cytokeratins and vimentin (Fig. 10 *a*, lane *d*). Fig. 10, *b* and *c* show a three-dimensional cord that developed after mixing suspensions of equal numbers of Cl 31 E and Cl 30 F cells. It is apparent that epithelial cells surrounding the central bundle of fibroblasts (Fig. 10 *b*) show a much stronger immunofluorescence with anti–cytokeratin LE-61 antibodies (Fig. 10 *c*) than neighboring Cl 31E cells directly adhering to the cell culture plastic.

Discussion

We report here the establishment and characterization of the spontaneously immortalized, mouse mammary cell line IM-2 and its epithelial and fibroblast-like clonal subpopulations. Even after 8 mo of continuous culture, IM-2 and its clones remained nontumorigenic in nude mice. The central finding of this report is that a mammary epithelial cell clone when provided with a permissive fibroblastic environment, acquires organized structures and differentiated functions corresponding to those for which the cells were originally determined in vivo. Coculture of a clonal mammary epithelial cell line with fibroblastic cells resulted in the formation of structures in which the deposition of laminin, the reorganization of intermediate filaments, and the competence to produce casein coincide. These findings give insight into a sequence of events occurring after epithelial–fibroblastic cell contact that have not previously been documented.

Dependent on the mode of coculture, two different types of structures developed, both of which allowed the free arrangement of mammary epithelial and fibroblastic cells. Both coculture procedures generated organized structures only after the unphysiological culture plastic had been saturated by either epithelial or fibroblastic cells. When cell suspensions were mixed, three-dimensional cords developed, allowing fibroblastic cells to organize themselves into condensed bundles on top of a plastic-adherent epithelial monolayer. These bundles, in turn, provide a new substrate for epithelial cells that envelope the fibroblastic core (Fig. 4, *d* and *e*). According to the same interactive principle, when epithelial cells were added to a preexisting confluent fibroblastic
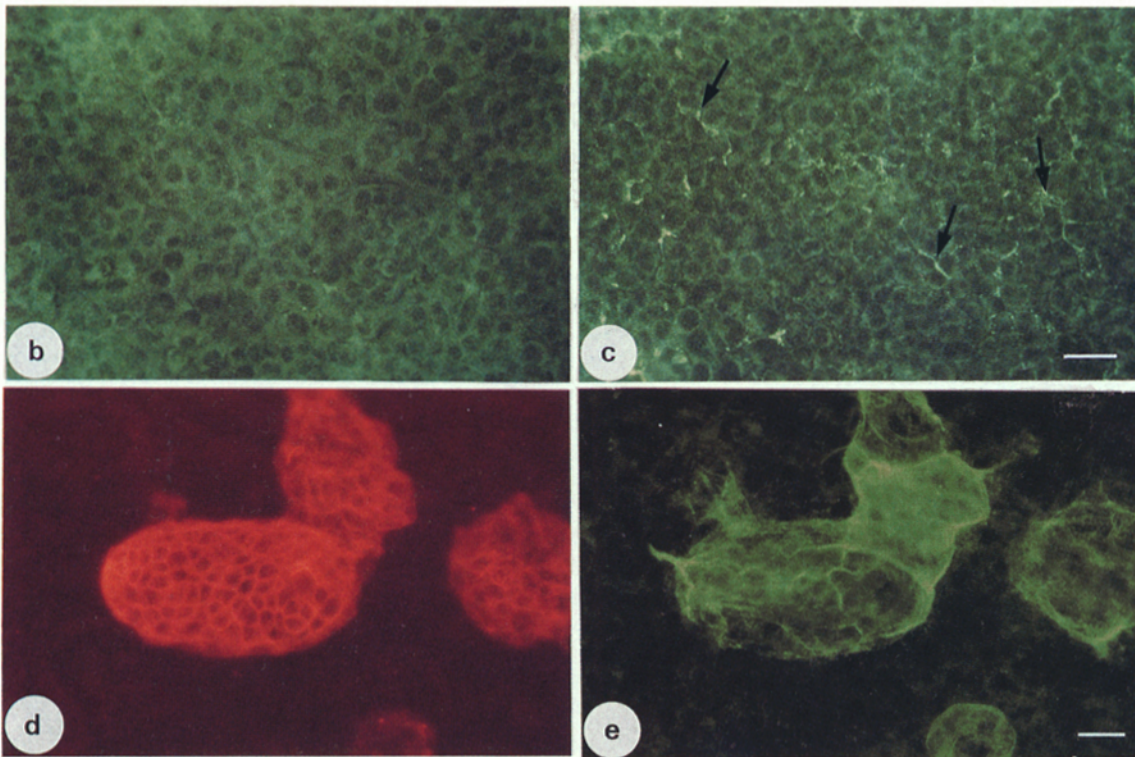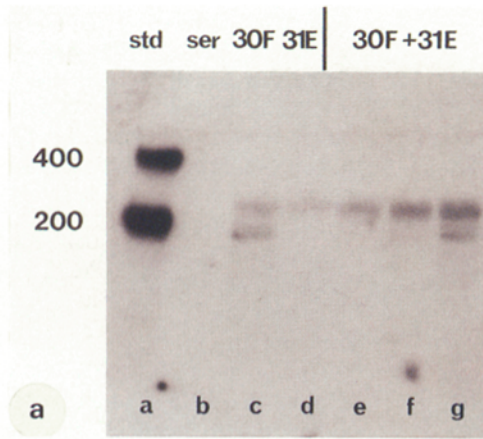
Figure 9. (*a*) A polyclonal rabbit antiserum recognizing mouse laminin was used to show laminin subunits released into the culture medium by Cl 30F and Cl 31E cells and by cocultures of the two. Lane *a* demonstrates laminin subunits (A chain = ∿400 kD; B chains = ∿200 kD) isolated from Engelbreth-Holm-Swarm tumor. Lane *b* shows a sample of fresh culture medium used in the experiment that contained 1% FCS. Lane *c* indicates the laminin B1 and B2 chains detectable in the medium of Cl 30F cultures. Lane *d* illustrates the laminin B1 chain found in culture medium of Cl 31E cells. Lanes *e*, *f*, and *g* demonstrate the simple additive accumulation of laminin B1 and B2 subunits when constant numbers of Cl 31E cells (10^6) were mixed with increasing numbers of Cl 30F cells (10^5, lane *e*), (5×10^5, lane *f*), and (10^6, lane *g*). (*b*) Immunofluorescence indicates laminin-specific staining of Cl 30F cells. Diffuse cytoplasmic staining is consistent with their synthesis and secretion of laminin B1 and B2 chains. (*c*) Deposited laminin fibrils (*arrows*) are sporadically distributed on the surfaces of a confluent monolayer of Cl 31E cells. (*d*) Clusters of Cl 31E cells on top of a confluent monolayer of Cl 30F cells stained by the cytokeratin-specific antibody LE-61. (*e*) The same field as in *d* stained with an antibody recognizing the laminin–nidogen complex. Deposition of laminin occurs in areas where epithelial cell clusters are in intimate contact with the fibroblastic layer. Bars: (*b* and *c*) 40 μm; (*d* and *e*) 20 μm.

monolayer, only flat epithelial cell clusters developed (see Fig. 7).

When plated on a formaldehyde-fixed confluent monolayer of fibroblastic Cl 30F cells, Cl 31E cells still responded efficiently to lactogenic hormones. Thus, it can be excluded that junctional complexes formed between the two cell types, or soluble factors released by the fibroblastic cells are necessary to mediate differentiated functions of the mammary epithelial cells. More probable is that stromal extracellular matrix components such as fibronectin and collagen type I
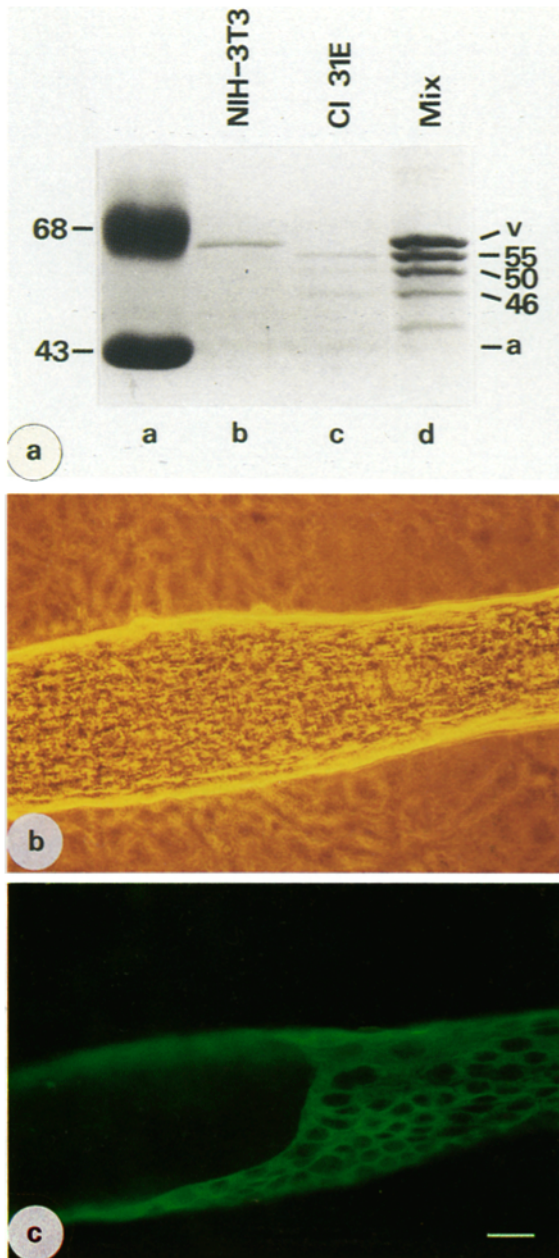
Figure 10. (*a*) SDS-PAGE of detergent insoluble cell extracts. The amounts of protein loaded onto the gel were normalized with respect to the total detergent soluble protein that could be extracted from each sample. BSA (68 kD) and phosphoglycerate kinase (43 kD) are molecular mass markers (lane *a*). Vimentin (58 kD) is present in the extract of NIH-3T3 fibroblasts (lane *b*). Three bands corresponding to cytokeratins of 55, 50, and 46 kD, prepared from Cl 31E cells are barely evident (lane *c*). Coculture of the two cell types in a ratio of 1:1 results in the greatly increased accumulation of detergent-insoluble intermediate filament proteins (lane *d*). (*b*) Phase-contrast micrograph of a three-dimensional cord consisting of centrally located fibroblastic Cl 30F cells surrounded by mammary epithelial Cl 31E cells. (*c*) The same fibroblastic bundle shown in *b* is surrounded by a partially complete tubular wall of epithelial cells as visualized using the cytokeratin-specific antibody LE 61. Epithelial cells directly surrounding the fibroblasts show greatly increased expression of cytokeratins as compared to the epithelial monolayer adherent to cell culture plastic. Bar, 20 μm.

located at the surface of fibroblastic cells are involved in establishing the competence of the Cl 31E cells. In fact, we found that Cl 31E cells in monoculture were competent to synthesize appreciable amounts of casein when grown on collagen type I gels alone (see Fig. 8 *b*, lane *b*). It is tempting to conclude from these data that extracellular matrix components deposited by mesenchymal cells provide the initial structural basis required for the deposition of a basal lamina.

In view of the fact that fibroblast-like Cl 30F cells release both B chains of laminin, while epithelial Cl 31E cells produce only the B1 chain, the question arises to what extent are the laminin chains synthesized by fibroblast-like cells involved in basal lamina composition. Klein et al. (1988) showed that both B chains were seen even in (uninduced) kidney mesenchyme. Furthermore, Simon-Assmann et al. (1988), taking advantage of chick-rat epithelio- mesenchymal tissue recombinants, clearly showed that the mesenchymal compartment can be the principal endogenous source of the basal lamina constituent collagen type IV. It is therefore probable that the laminin chains produced by Cl 30F cells become integrated in the basal lamina formed between epithelial and fibroblastic cells. The finding that neither Cl 30F cells nor NIH 3T3 fibroblasts showed laminin deposition in monoculture, while Cl 31E cells exhibited this potential, indicates that it must be the epithelial cells that express some function, i.e., laminin receptors, or possibly protein cross-linking enzymes required for the local fixation of laminin. Our observation that areas of deposited laminin colocalized with clusters of Cl 31E cells (see Fig. 9, *d* and *e*) in which almost all of the cells could be induced to synthesize caseins (see Fig. 7) is consistent with the idea that a basal lamina is required for mediating epithelial cell differentiation. Culturing mammary epithelial cells on floating collagen gels (Emerman and Pitelka, 1977; Lee et al., 1985), on nitrocellulose filters (Parry et al. 1987), or as is demonstrated in this communication, in association with a basal lamina renders the cells competent to synthesize and secrete caseins. Since the only conceivable feature common among these three matrices is that of a permeable physical support, it seems possible that the accessibility of the basolateral cell surface markedly influences epithelial cell differentiation. It has been demonstrated by various groups that epithelial cells form monolayers with a higher degree of differentiation when the basolateral surface is directly accessible to the growth medium (Handler et al., 1984; Fuller et al., 1984; Simons and Fuller, 1985; Parry et al., 1987). One of the major functions of the basal lamina therefore might be to mediate the access of nutrients, hormones, and growth factors to an area of the polarized cell surface where the respective receptors are localized.

There is, furthermore, a growing body of evidence suggesting that the basal lamina constituent laminin is involved in reorganizing the epithelial cytoskeleton (Sugrue and Hay, 1986; Blum and Wicha, 1988). The intact cytoskeleton is in turn thought to bind polysomes and mRNA, thus influencing transcription and/or stabilization of specific mRNA species (Bonneau et al., 1985; Walker and Whitfield, 1985; Ornelles et al., 1986). We demonstrate here that cytokeratin intermediate filaments, known to be associated with the cytoskeleton in epithelial cells, are greatly increased in Cl 31E cells cocultured with fibroblastic cells. In contrast, there were only small amounts of these proteins detectable when

Cl 31E cells were grown in monocluture on plastic. Data obtained by Ben Ze'ev (1984) for the Madin–Darby bovine kidney (MDBK) cells suggest that the rate of cytokeratin synthesis is mediated by cell density. Our data, however, indicate that Cl 31E cells showing cytokeratin protein accumulation were located exclusively in areas of laminin deposition (see Fig. 9, d and e). Thus, we regard it as likely that it is the presence of a basal lamina that regulates the formation and/or stabilization of cytokeratin intermediate filaments.

Cl 31E cells showing high levels of cytokeratin proteins also coincided locally with the cells synthesizing caseins (see Figs. 5 and 7). This finding raises the possibility of a functional role for cytokeratin intermediate filaments in epithelial cell differentiation. It has been postulated that cytokeratins play some as yet undefined role in the construction of the cytoskeleton (for review see Lazarides, 1980). On the other hand, evidence has been adduced which makes this increasingly doubtful (Klymkowsky, 1981; Lin and Feramisco, 1981). Data obtained by Traub et al. (1982) suggest that, under physiological ionic conditions, the fibroblastic intermediate filament polypeptide vimentin is able to interact both with RNA and single-stranded DNA. They postulate that vimentin and/or its derivatives might be involved in transcriptional and posttranscriptional control. There is some evidence that like vimentin, cytokeratins have nucleic acid binding properties.

In this study we have demonstrated that a defined mammary epithelial cell clone which stably retains its morphological homogeneity can be manipulated to generate differentiated structures and functions when cocultured with fibroblastic cells or, to a lesser extent, when grown on collagen type I gels. Our data support the model that intimate epithelial-fibroblastic cell contact is a prerequisite for the formation of a basal lamina that mediates cytoskeletal reorganization and access of macromolecules to the basal surface of epithelial cells. Both are likely to be involved in rendering the epithelial cells competent to differentiate functionally in response to lactogenic hormones. The nontumorigenic cell clone Cl 31E should prove useful for studying epithelial-mesenchymal cell interactions, structural and functional differentiation of mammary epithelium, and for examining the influence of oncogenes upon these processes.

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