Cell Proliferation and Milk Protein Gene Expression in Rabbit Mammary Cell Cultures

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ABSTRACT We analyzed the synthesis of DNA, the rate of cell proliferation, and the expression of milk protein genes in mammary cells grown as primary cultures on or in collagen gels in chemically defined media. We assessed DNA synthesis and cell growth, measured by [^{3}H]-thymidine incorporation into acid-insoluble material, DNA content, and cell counts, in a progesterone- and prolactin-containing medium. In some experiments, cultures were pulsed for 1 h with [^{3}H]thymidine and dissociated into individual cells which were cytocentrifuged and processed for immunocytochemistry and autoradiography. We analyzed expression of milk protein genes at the transcriptional, translational, and posttranslational levels in a progesterone-depleted medium in the presence or absence of prolactin. We measured protein secretion by radioimmunoassays with antisera directed against caseins, α-lactalbumin and milk transferrin. We determined protein synthesis by incorporating radio-labeled amino acids into acid-precipitable material and by immunoprecipitating biosynthetically labeled milk proteins. We assessed the accumulation of casein mRNA by hybridizing total cellular RNA extracted from cultured cells with ^{32}P-labeled casein cDNA probes. On attached collagen gels, the cells synthesized DNA and replicated until they became confluent. The overall protein synthetic activity was low, and no milk proteins were synthesized or secreted even in the presence of prolactin. The block in milk protein gene expression was not restricted to translational or posttranslational events but also included transcription, since no casein mRNA accumulated in these cells. On floating gels, protein synthesis was threefold higher than in cells from attached gels. Overall protein synthesis as well as casein and α-lactalbumin synthesis and secretion were prolactin-dependent with maximal stimulation at around 10^{-9} M. A marked inhibition occurred at higher hormone concentrations. Casein mRNA accumulated in these cells, provided prolactin was present in the medium. In contrast, these cells did not synthesize DNA, nor did they replicate. In embedding gels, the rate of cell proliferation was exponential over 25 d with a doubling time of ~70 h. The overall protein synthesis increase was parallel in time with the increase in cell number. Caseins and α-lactalbumin (in contrast to transferrin) were synthesized only in the presence of prolactin. We observed the same hormone dependency as with cells growing on floating gels. The number of casein- and transferrin-positive cells was measured after dissociating the cell cultures. At day 12, 60% of the total cells stored transferrin in small cytoplasmic vesicles, whereas only 25% of the cells accumulated casein. Differences in the organization and in the shape of mammary cells depending on cell surface conditions suggest that the geometry of the cells, their interaction with extracellular matrix constitutents, and cell-to-cell interactions play a role in the expression of two mammary functions: DNA synthesis and growth, as well as milk protein gene expression.

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MAY 1983 1435-1442

The mechanisms regulating DNA synthesis and cell replication during pregnancy are poorly understood. It is known from in vitro studies that estrogens in conjunction with progesterone induce DNA synthesis in end-bud cells, whereas duct cells seem to respond preferentially to progesterone (3). Ovarian steroids, however, are not the only hormones involved in the stimulation of mammary gland growth. Adrenal steroids, insulinlike hormones, and thyroid and lactogenic hormones have been shown to induce lobulo-alveolar differentiation in rat and mouse (29, 43, 44). Their role has been examined in vitro by means of growing organ explants of mammary gland in chemically defined media supplemented with insulin, gluco- or mineralocorticoids, and prolactin used at pharmacological concentrations (18, 19). Since growth occurred in tbe absence of ovarian steroids, it has been speculated that endogenous levels of estrogen and progesterone carried over into the culture system might be sufficient for priming the tissue, thus rendering it independent from exogenous addition of these hormones. In the rabbit, there is a lack of information concerning tbe hormonal environment that initiates and sustains those physiological changes.

The onset of milk protein gene expression and milk secretion at parturition and during lactation is induced and modulated by an interplay among a complex of hormones including steroid and polypeptide hormones that appear to act in a specific temporal sequence (36). The regulation of milk protein gene expression by lactogenic hormones has been extensively analyzed at the molecular level with complementary DNA hybridizing with casein and α-lactalbumin mRNAs as probes. Several species have been studied both in vivo and in vitro, using organ explants. It is now well established in rabbit (9, 16, 17), rat (13, 33, 34, 36), mouse (1, 5, 40), and guinea pig (6) that lactogenic hormones, i.e., prolactin, placental lactogens, and primate growth hormones, regulate the gene expression of the caseins and of α-lactalbumin—a protein involved in lactose synthesis. In rat mammary organ cultures, prolactin exerts pleiotropic effects by increasing on the one hand casein transcription and on the other hand the half-life of newly synthesized mRNA molecules (13). Progesterone inhibits both these transcriptional and posttranscriptional events (17, 35), whereas glucocorticoids, which are inactive alone, potentiate the effects of lactogenic hormones (8). There have been few studies reported on the regulation of milk protein gene expression at the transcriptional level either in primary mammary cell cultures or in established cell lines (41). Most studies using primary cultures of normal or transformed cells were restricted to translational or post-translational events. In such systems, the amount of casein and α-lactalbumin synthesized or secreted per cell remained far below the amounts produced in the intact lactating gland (12, 30, 31).

Following the work of Pitelka and of her co-workers (11, 12, 45), we grew mammary cells in or on collagenous matrices in serum-free chemically defined media, and we examined the role of cell shape and cytodifferentiation on the functional differentiation of mammary cells by morphological means (14). In the present paper, we analyze cell proliferation and DNA synthesis as well as milk protein gene expression at the transcriptional, translational, and posttranslational levels under three different culture conditions, i.e., on attached, floating, or embedding collagen gels. We show that cell replication and milk protein gene expression depend both on a specific hormonal environment and on cell organization. Part of this work has been presented in an abstract form (23).

MATERIALS AND METHODS

Materials are given in the accompanying paper (14).

Cell Sampling: Pools of dispersed mammary cells were routinely prepared from three individual rabbits, frozen, and stored in liquid nitrogen. To normalize our experimental data, prior to the exhaustion of one pool, we took a sample for comparison with the subsequent pool.

Culture Conditions: The standard medium for plating, growing, and biosynthetically labeling the cells was a 1 to 1 mixture of M199 and F12 medium supplemented with gentamicin (100 μg/ml) and fungizone (2.5 μg/ml) for the first 5 d of culture. Cell aggregates (see Materials and Methods of accompanying paper [14]), suspended in medium containing 20% horse serum and 5% fetal calf serum, were plated at a density of 10^4 cells/cm^2 for cell proliferation and DNA synthesis studies, and at 5×10^5 cells/cm^2 for milk protein gene expression analysis. The cells were embedded at a density of 10^5/ml of collagen mixture for cell replication and at 10^6 cells/ml for milk protein gene expression experiments. The cells were cultured for 24 h in the same serum containing medium and then in serum-free medium complemented with 0.25% bovine serum albumin, 10^{-10} M dexamethasone, 10^{-10} M 17-β-estradiol, 10^{-9} M triiodothyronine, 10 ng/ml epidermal growth factor, and prostaglandin F2α (PGF2α), and 5 μg/ml insulin. For sustained growth and DNA synthesis, the medium was supplemented witb 10^{-8} M progesterone and 5×10^{-9} ovine prolactin (PRL) unless otherwise stated. For protein synthesis and secretion experiments, we removed progesterone and added PRL, at concentrations varying between 10^{-6} and 10^{-10} M, to determine the dose-dependency. The medium was changed every day. Floating gels were obtained 2 or 5 d after plating by mechanically detaching the attached collagen gels with a Pasteur pipet.

Protein Synthesis: The attached, floating, and embedding gels were digested for 2 h at 37°C, with purified collagenase (see Materials and Methods of accompanying paper [14]). For immunoprecipitation studies, the cell layers or the outgrowths of six wells (35 mm in diameter) were pooled for each experimental determination, washed in methionine-free medium, and incubated with 2.5 ml of [^{35}S]methionine (>1,200 Ci/mmol) at 200 μCi/ml. For assessment of overall protein synthesis by precipitation of acid-insoluble material, the cell layers and the outgrowths of two wells (16 mm in diameter) were pooled, washed in leucine-free medium, and incubated with 0.5 ml of [^{3}H]leucine (>300 Ci/mmol) at 10 μCi/ml. At tbe end of incubation, protease inbibitors were added at a final concentration of 1 mM for phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml for pepstatin A, leupeptin, antipain, and aprotinin and 10 μg/ml for soybean trypsin inhibitor. The cells were centrifuged and the supernatant was saved. 1–2 ml of H_2O were added to the pellets, which were sonicated for 30 s at 4°C. Aliquots were used to measure the DNA content (21) and to determine the incorporation of radioactivity into acid-insoluble material, or for immunoprecipitation with anticaseins, anti-α-lactalbumin, and antitransferrin sera (7) according to Maccechini et al. (26). Samples of the medium (supernatant), the pellet, and the immunoprecipitates were analyzed by SDS PAGE (27), followed by fluorography (25).

Protein Secretion: The medium from cells grown on attached or floating gels was collected each day. The gels were then digested, and the cells were recovered and lysed as described. The amount of milk protein (caseins α, κ, α-lactalbumin, and transferrin) was measured by radio-immunoassays using specific goat antisera (7), radio-iodinated purified milk proteins, and a sheep anti-rabbit F(ab')$_2$ serum to precipitate the soluble immune complexes in a classical sandwich assay. The amount of secreted protein we estimated from standard curves established with purified cold milk proteins (7) and the values were normalized to the DNA content and hence to the number of cells, assuming 7 pg DNA per cell (22), and expressed as nanograms of milk protein secreted per 24 h per 10^6 cells. To determine protein accumulation in cells from embedding gels, the cells were dissociated with trypsin (2.5 μg/ml), cytocentrifuged, and labeled with anti-milk protein sera.

DNA Synthesis and Cell Counts: The collagen gels were first digested with collagenase for 1–3 h. The cells were then incubated at 37°C with 2 μCi/ml of [^{3}H]thymidine for 1 h. The amount of ^{3}H in TCA-insoluble material was counted in a liquid scintillation counter, and the values were normalized to DNA content. For cell counting, the cells were further treated with trypsin (2.5 μg/ml) and EDTA (1 mM) for 15 min, dispersed with a siliconized Pasteur pipet, and counted in a hemocytometer. Some cells were cytocentrifuged and processed for autoradiography or immunocytochemistry as described below.

Extraction of RNA and Blot Analysis: Total cellular RNA was extracted at 65°C with phenol (37) from cells grown on attached, floating, or in embedding collagen gels. Total cellular RNA was denatured in 1 M glyoxal, 50% DMSO, 10 mM phosphate, pH 6.5, 0.1% SDS for 5 min at 50°C (following a modification of the procedure of McMaster and Carmichael [28]) and was electrophoresed on a horizontal agarose slab gel in 10 mM phosphate buffer, pH 6.5. The RNA was transferred onto nitrocellulose filters following the protocol of

Thomas (42) and was hybridized to cloned ^{32}P-labeled α-casein cDNA (39). Hybridization with polyadenylated RNA extracted from lactating mammary gland served as a control.

Immunofluorescence: Mammary cells recovered from collagen gels and labeled for 1 h with [^{3}H]thymidine were cytocentrifuged on glass slides, fixed briefly in ether-ethanol (1:1), rehydrated with Phosphate-buffered saline (PBS), and reacted with goat anti-rabbit milk protein sera (7), diluted 1:10 (antibody titer ~0.05–0.1 mg/ml). The slides were washed three times in PBS, exposed for 15 min to biotinylated sheep F(ab')$_2$ directed against goat IgG, and finally incubated with lissamine- or FITC-labeled streptavidin (R. Rodewald, D. Papermaster, and J. P. Kraehenbuhl, manuscript in preparation). The slides were then washed three times in PBS and once in distilled water, and were processed for autoradiography with liquid Ilford L4 emulsion. The autoradiograms were exposed for 2 wk, developed, and then observed in a Zeiss photomicroscope II with a fluorescent attachment and equipped with Osram HBO 100-W high pressure mercury vapor light source, BG12 excitation filter, and BG38 suppression filter.

RESULTS

DNA Synthesis and Cell Proliferation

DNA synthesis assessed by [^{3}H]thymidine incorporation into acid-insoluble material and growth rates estimated from cell counts and DNA content were determined for cells grown under the three-culture conditions: attached, floating, and embedding gels. In a first group of experiments, we measured the rate of cell growth and DNA synthesis for cells embedded in collagen and cultured either in the presence of progesterone (10 ng/ml), PRL (5×10^{-9} M), or both hormones, and compared to that of cells grown in the presence of optimal concentrations of serum. We determined the dependency on serum concentration independently and found it to be 20% horse serum and 5% fetal calf serum (data not shown). In the absence of progesterone and PRL DNA synthesis was minimal and the number of cells in embedding gels slowly decreased with time (data not shown). In contrast, the addition of either progesterone or PRL promoted both DNA synthesis and cell proliferation (Fig. 1). The effect of these two hormones when added simultaneously was synergistic and exponential growth was observed for up to 16 d (Figs. 1 and 2), with a doubling time of ~70 h identical to that observed for cells cultured in serum-containing medium. Following the embedding of spheroids into the collagen matrix, there was a significant decrease in the number of cells during the first days of culture and a low rate of DNA synthesis. This cell loss was more pronounced for cultures grown in serum-free media than in serum-containing medium (Fig. 1). Interestingly, there was no fibroblast proliferation under serum-free conditions as opposed to cultures grown in serum-containing media.

On attached collagen gels, the rate of cell proliferation in the presence of progesterone and PRL was much slower than in embedding gels, with a doubling time of ~120 h. At confluency, this initial proliferation was followed by a plateau phase, with little DNA synthesizing activity (Fig. 2, *upper* panel). From day 6 onward, the confluent cell monolayers started to retract, leaving cell-free areas of collagen gels. The appearance of such holes was followed by an increase in DNA synthesis and a new wave of cell replication (Fig. 2, *upper* panel).

When attached gels were freed from tbe plastic wells on day 5, they retracted and formed floating collagen gels. Upon detachment, DNA synthesis progressively decreased and remained low until the end of the experiment. Similarly, the number of cells which increased as long as the gels were attached (day 5), decreased upon detachment and then remained constant (Fig. 2, *middle* panel).

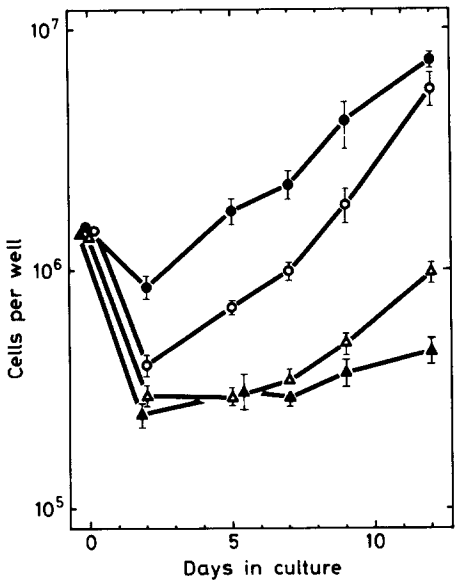

FIGURE 1 Growth rates and DNA synthesis of rabbit mammary cells grown in embedding collagen gels. Mammary cells (10^6 cells/ml) were embedded in 10-cm^2 plastic wells (2.5 ml collagen solution) and cultured in F12/M199 medium supplemented with insulin, EGF, PGF2α, dexamethasone, triiodothyronine, and 17-β-estradiol at concentrations given in Materials and Metbods. PRL (5×10^{-9} M) (▲), progesterone (10^{-8} M) (△), or progesterone (10^{-8} M) and PRL (5×10^{-9} M) (○), or 20% horse serum and 5% fetal calf serum (●) were added to the chemically defined medium. Values of cell numbers represent the mean of three independent experiments, plus or minus standard error of the mean (SEM).

Milk Protein Expression

PROTEIN SECRETION: We assessed accumulation of proteins in mammary cells grown on attached or floating gels and the secretion of proteins into medium by radio-immunoassay using monospecific antisera directed against casein (α or κ), α-lactalbumin, and transferrin. Since the apical membrane of the mammary cells faced the medium as a consequence of the reestablishment of cell polarity, we expected secreted proteins to be vectorially discharged into the medium, where their concentration could be determined. In contrast, since cells embedded in collagen formed luminal spaces in the center of ductlike structures, it was unlikely that secreted material would accumulate in the medium, but rather would be sequestered in the lumen and hence not readily available for measurements. Therefore, we determined accumulation of secreted material in embedded cells morphologically by immunofluorescent labeling of dispersed and cytocentrifuged cells. On attached gels secretion of protein by mammary cells remained low and undetectable during the entire 16-d experimental period (Fig. 3, *upper* panel): upon detachment of the collagen gel and rearrangement of the cells, both the amount of protein stored in the cells and of that secreted into the medium over a 24-h period started to progressively increase after a 24-h lag. The increase was continuous for casein (Fig. 3, *upper* panel), whereas a plateau was reached after 2 d for transferrin (Fig. 3, *lower* panel), and after 8 d for α-lactalbumin (Fig. 3, *middle* panel). Secretion of casein and α-lactalbumin was induced only in the presence of PRL which was not necessary for induction of transferrin secretion. Secretion of casein and α-lactalbumin was hormone-dose-dependent, with maximal stimulation at around 10^{-9} M and marked inhibition at higher concentrations. The maximum amount of protein

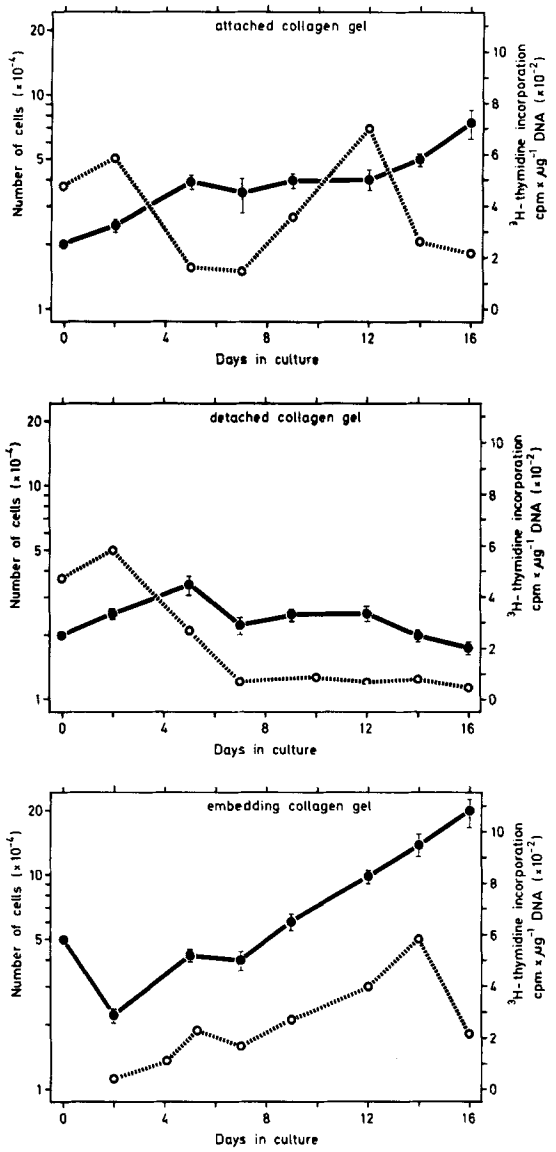

FIGURE 2 Growth rates and DNA synthesis of rabbit mammary cells grown on attached, detached (floating) and in embedding gels. Mammary cells were plated on collagen gels (2×10^4 cells/2 cm^2 wells) or embedded in collagen (5×10^4 cells in 0.5 ml collagen solution for 2 cm^2 wells) and cultured in chemically defined medium (see Materials and Methods) supplemented with progesterone (10^{-8} M) and PRL (5×10^{-9} M). Cell growth (——) was measured in three independent experiments, and the values of cell numbers represent the mean ± SEM. DNA synthesis, measured by the incorporation of [^{3}H]thymidine (1-h pulse with 2 μCi/ml) into acid-precipitable material, is expressed as cpms per μg of DNA (- - - - -).

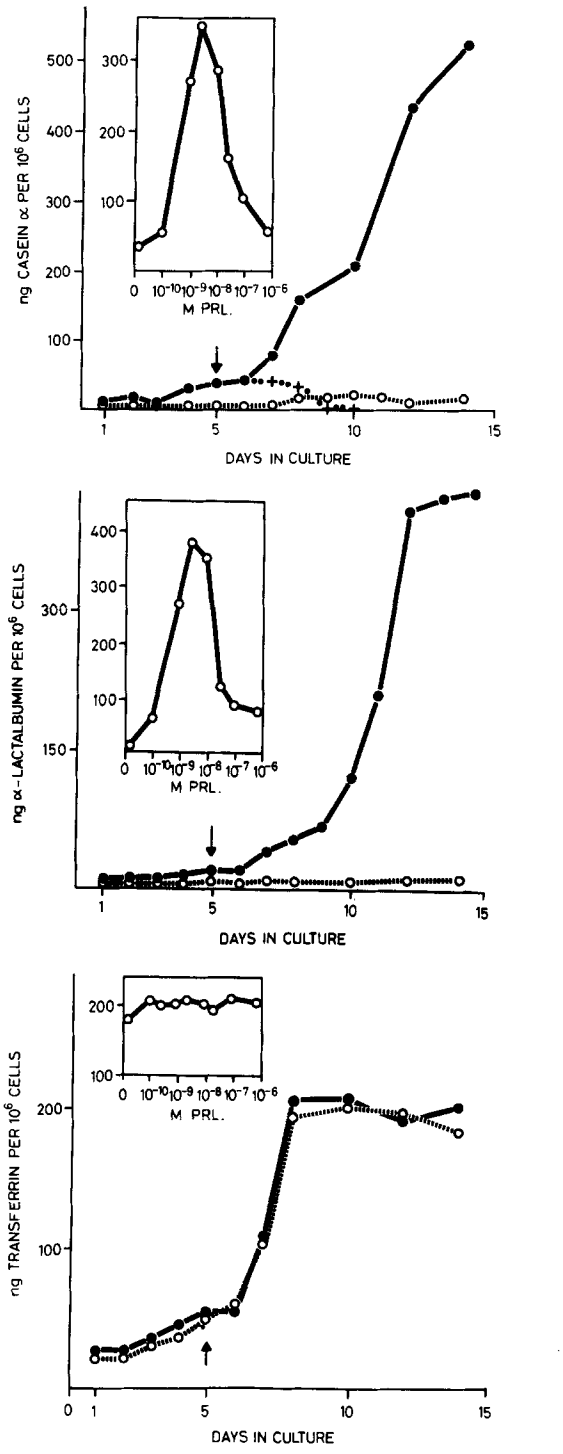

FIGURE 3 Secretion of milk proteins in rabbit mammary cells grown on attached or floating collagen gels. Secretion of casein α (*upper* panel), α-lactalbumin (*middle* panel), and transferrin (*lower* panel) was measured in the medium collected after 24 h by radio-immunoassay using monospecific antibodies (see Materials and Methods). The values are expressed as ng protein secreted per 24 h per 10^6 cells. The cells (10^6 cells/2 cm^2) were grown in F12/M199 medium supplemented with insulin (5 g/ml), dexamethasone (10^{-10} M), in the absence (○) or the presence (●) of PRL (10^{-9} M). At day 5 (arrow) the gels were mechanically freed from the plastic wells (floating gels). In one experiment (*upper* panel) secretion of casein was determined for cells cultured on attached gels (+). The PRL dependency was measured each day and the insets represent values determined on day 11.

secreted by 10^6 cells over a 24-h period was in the range of 600 ng for casein α, 500 ng for casein κ, 400 ng for α-lactalbumin, and 200 ng for transferrin.

Results from immunofluorescent labeling of cells recovered from embedding gels are given in Table I and Fig. 4. In these experiments, the cultures, after digestion of the collagen gel with purified collagenase, were pulsed for 1 h with [^{3}H]thymidine and further digested for 15 min with trypsin. The cells were then mechanically dispersed by pipetting the cultures, and finally were cytocentrifuged, labeled with anticasein α or antitransferrin antibodies and were processed for autoradiography. In culture, mammary cells accumulating transferrin increased in number with time to reach a plateau level around

TABLE I

Analysis of [^{3}H]Thymidine Incorporation and Transferrin or Casein Accumulation in Cytocentrifuged Mammary Cells from Embedding Collagen Gels

	Day 1	Day 4	Day 8	Day 11
Control				
^{3}H-TdR	30 ± 3	22 ± 2	10 ± 1	8 ± 1
Casein	0	0	1.5	0.5
Casein + ^{3}H-TdR	ND	0	0	0
Transferrin	1	24 ± 1	55 ± 4	61 ± 3
Transferrin + ^{3}H-TdR	ND	2.5 ± 1	2.5 ± 1	5 ± 2
PRL				
^{3}H-TdR	30 ± 3	24 ± 5	12 ± 21	9 ± 1
Casein	ND	8 ± 1.5	16 ± 3	25 ± 8
Casein + ^{3}H-TdR	ND	<1	<1	1
Transferrin	ND	21 ± 5	53 ± 5	57 ± 8
Transferrin + ^{3}H-TdR	ND	4 ± 0.5	3 ± 1	4 ± 1

On indicated days, the collagen gels were digested, the monolayers were incubated for 1 h. with 2 μCi/ml of [^{3}H]thymidine, the mammary cells dispersed by trypsin-EDTA treatment and cytocentrifuged. After immunocytochemical and autoradiographic processing, the labeled cells were counted and the numbers expressed as percent of labeled cells to total cells ± standard error of the means. Three experiments were run in parallel with the same starting frozen cells.

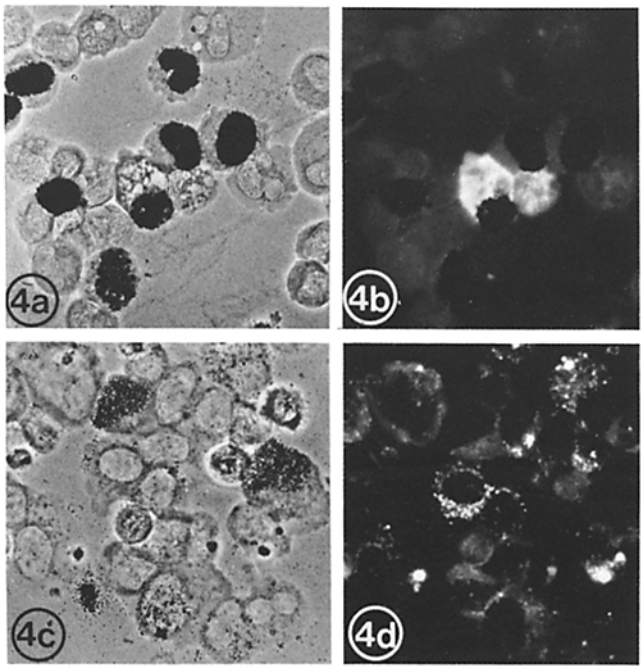

FIGURE 4 Accumulation of milk proteins in mammary cells grown in embedding gels. Mammary cells embedded in collagen (see Materials and Methods) were grown in F12/M199 medium supplemented with insulin, epidermal growth factor, PG2α, dexamethasone, triiodothyronine, and 17-β-estradiol at concentrations given in Materials and Metbods and with 10^{-9} M PRL. At day 8, the collagen gels were digested with purified collagenase. The cultures were pulsed for 1 h with 2 Ci/ml [^{3}H]thymidine and the cells trypsinized and cytocentrifuged. They were then reacted with biotinylated goat anticasein antibodies (*a* and *b*), or with biotinylated goat antitransferrin antibodies (*c* and *d*), and with lissaminated streptavidin. The cells were then coated with Ilford L4 emulsion, and the autoradiograms were exposed for 2 wk and then were developed (*a* and *c*). (*a* and *c*) Phase contrast. (*b* and *d*) Immunofluorescence. × 800.

day 11, with ~60% of positive cells. The increase in number was independent of the presence of PRL. In contrast, we detected casein α in cells only when the cultures were grown in the presence of PRL. The number of casein-positive cells also increased with time but did not exceed ~30% of the total cells at day 11. Only a few cells storing milk proteins were labeled with [^{3}H]thymidine. ~5% of the transferrin-positive cells had labeled nuclei, and ~1% of the casein-positive cells were double-labeled.

Synthesis and Storage of Milk Proteins

Protein synthesis measured by the incorporation of radioactive amino acids into TCA-insoluble material recovered from both the medium and the mammary cells was determined in attached or floating collagen gel cultures. Since secreted material was sequestered in the luminal space when cells were grown in embedding gels, only total incorporation into both secreted and cellular proteins was determined in this case. The rate of synthesis was low in cells cultured on attached gels, and low amounts of radio-labeled secreted proteins were released into the medium for periods up to 15 d (Fig. 5). In contrast, protein synthesis and secretion of radioactive proteins increased 2.5-fold upon collagen gel detachment (day 5). A more than threefold increase over controls was achieved when prolactin was added at optimal concentration (10^{-9} M). The overall synthetic activity was again hormone-dose-dependent with a maximal stimulation at a concentration between 5×10^{-10} and 5×10^{-9} M and a marked decrease in the rate of synthesis at higher concentrations (Fig. 5), as already shown for the accumulation and secretion of milk proteins. When cells were embedded in collagen, tbe overall protein synthetic activity remained low during the first few days; then, as the number of cells increased, there was a continuous hormone-dependent increase in the incorporation of [^{3}H]leucine expressed as cpm per μg DNA until a steady state was reached around day 10. From day 10 on, the amount of incorporated [^{3}H]leucine (cpm/μg DNA) was similar to that found for floating gels with a threefold increase in PRL-stimulated cells over controls. To identify which milk proteins were biosynthetically labeled and controlled by prolactin, cells grown on attached, floating, or in embedding gels for 8 d were pulsed for 3 h with [^{35}S]methionine. The labeled cells were lysed in the presence of SDS and analyzed by SDS PAGE. Milk proteins were immunoprecipitated from the cell lysate with anticasein α and antitransferrin

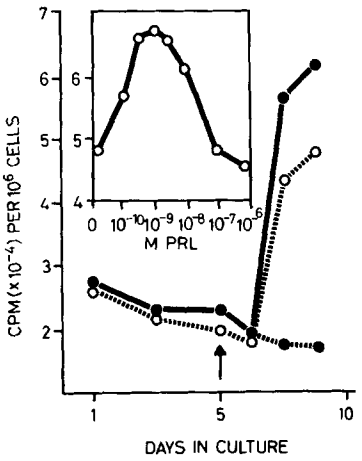

FIGURE 5 Protein synthesis in mammary cells grown on attached or floating collagen gels. Mammary cells (10^6/2 cm^2) were plated and grown in the same medium as described in Fig. 4 in the absence (○) or presence (●) of PRL hormone (5×10^{-9} M). Some gels were detached on day 5, and some remained attached (●···●). At the indicated time, the collagen gels were digested, and the cells were incubated twice for 10 min in the same medium without leucine and then for 3 h in the same medium containing 10 μCi/ml [^{3}H]leucine. Values are expressed as cpms per 10^6 cells. The PRL-dependency was determined on day 7.

antibodies. The results of such an experiment, illustrated in Fig. 6, indicate that the synthesis of transferrin and of casein α occurred only on floating and embedding gels. Whereas the synthesis of transferrin is PRL-dependent, the synthesis of caseins requires the presence of optimal concentrations of PRL.

Accumulation of Casein mRNA

To test whether the block in milk protein gene expression observed in cells grown on attached collagen gels was restricted to translational or postranslational events, we assessed the accumulation of mRNA in cells grown on attached gels, as well as in cells cultured under the two other conditions, with cDNA specific for casein α. When total cellular RNA was extracted, electrophoresed, blotted onto nitrocellulose paper, and then hybridized with ^{32}P-labeled casein cDNA, two observations were made which are illustrated in Fig. 7. First, accumulation of casein mRNA occurred only in the presence of PRL, and second, there was no detectable casein mRNA in cells from attached gels, even in the presence of optimal PRL concentrations.

DISCUSSION

Two mammary cell functions, DNA synthesis and cell replication, that are required for the lobulo-alveolar differentiation of the mammary gland during pregnancy and milk protein gene expression at parturition and during lactation, have been maintained in nontransformed rabbit mammary cells cultured on two- or three-dimensional collagen matrices. In this study, the functional differentiation of mammary cells grown under the three culture conditions described in the accompanying paper (14) was analyzed biochemically in a chemically defined medium so that the hormone requirements for DNA synthesis, cell proliferation, and milk protein gene expression could be determined. Our results show that sustained growth and gene expression of milk proteins can be induced and maintained in mammary cells grown in three-dimensional stromal collagen matrices. In contrast, only one of these mammary functions is expressed in cells cultured on two-dimensional stromal collagen surfaces. On rigid substrates, the cells proliferate until they become confluent, but milk protein gene expression is inhibited at the transcriptional and translational levels, whereas on flexible substrates the cells are unable to grow but synthesize and secrete prolactin-insensitive and -inducible milk proteins.

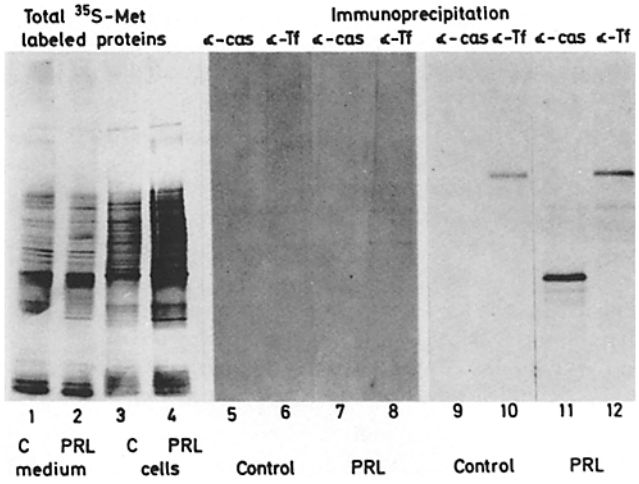

FIGURE 6 Immunoprecipitation of biosynthetically labeled milk proteins from mammary cells grown on attached, floating or in embedding collagen gels. Mammary cells were plated (5×10^6 cells/10 cm^2) or embedded (2.5×10^6 cells/2.5 ml collagen solution) in collagen and were cultured for 10 d in chemically defined medium as in Fig. 4, in the absence (control) or presence of 5×10^{-9} M PRL. At day 10, the collagen gels were digested, and the cultures were incubated twice for 10 min in medium without methionine, and then for 3 h in medium containing 200 μCi/ml of [^{35}S]methionine. The cultures were then centrifuged, and the pellets (cells) and the supernatant (medium) were collected. Lanes 1 to 4 represent [^{35}S] methionine labeled total proteins from cells grown on detached gels (lanes *3* and *4*) or from the medium recovered from the cultures (lanes *1* and *2*). Lanes 5 to 12 represent immunoprecipitation patterns of pellets (cells) using anticasein α (α-cas) or antitransferrin (α-*Tf*) antibodies. (Lanes *5–8*) Samples from attached collagen gels. (Lanes *9–11*) Samples from detached gels. The patterns obtained from samples of embedded cells were similar to the ones from floating gels.

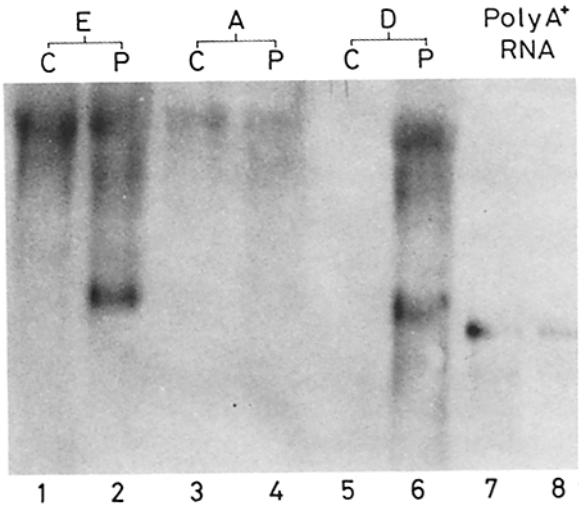

FIGURE 7 Extraction of RNA from mammary cells grown on attached, floating, or embedding collagen gels and blot analysis. Mammary cells were plated or embedded in collagen gels as described in Fig. 6. The cells were cultured for 8 d in the presence (*P*) or absence (*C*) of 5×10^{-9} M PRL. RNA was extracted, and blot analysis was performed as described in Materials and Methods. Polyadenylated RNA (poly [A$^+$] RNA) extracted from lactating glands served as controls (lanes *7* and *8*). (Lanes *1* and *2*) Samples from embedding gels (*E*). (Lanes *3* and *4*) Samples from attached gels (*A*). (Lanes *5* and *6*) Samples from floating gels (*D*). The slow migrating species detected in all samples represent poorly denatured molecules sticking to DNA which was not digested in these experiments.

Hormonal Requirements for Cell Proliferation

Growth and differentiation of the mammary gland during pregnancy depend both on pituitary and ovarian hormones (29). In organ explants or cell cultures, however, these hormones had little or no effect on mammary cell proliferation (43). The use of primary mammary cell cultures for the analysis of cell growth has been limited to studies of [^{3}H]thymidine incorporation in short-term cultures, because of the inability to maintain mammary cells in long-term cultures. Due to recent developments in the cultivation of mammary cells using stromal collagen matrices (45), it became possible to maintain sustained growth and thus to determine the effect of various hormones and growth promoting factors by estimating the increase in cell number (20, 32, 46).

We compared the growth rate of rabbit mammary cells grown under the three-culture conditions described above in a

serum-free medium to that in a serum containing medium. The optimal serum conditions were similar to those determined for mouse mammary epithelial cells (15). The conditions for the growth of a human mammary tumor cell line in a serum-free medium containing physiological concentrations of insulin, transferrin, epidermal growth factor, PGF2, and fibronectin (2) provided only minimal cell growth for rabbit mammary cells cultured in embedding collagen gels. The medium we selected contained PGF2α, and insulin, but included also glucocorticoids, progesterone, and 17-β-estradiol added at concentrations identical to those found in rabbit at midpregnancy (38). We added thyroid hormones that influence lobulo-alveolar differentiation in vitro (44) at physiological concentrations, as well as PRL at concentrations slightly above those required to saturate half the receptors on mammary cells (38). In the absence of PRL and progesterone, DNA synthesis and cell proliferation were significantly lower than in serum-containing medium. Added singly, these hormones promoted growth, but together they had a synergistic effect with growth rates similar to those obtained with serum.

Finally, fibroblast domination of primary mammary cultures, which is a serious problem in rabbit mammary cultures, was minimized in this chemically defined medium. Although mammary epithelial cells are highly enriched by isopycnic centrifugation and cell-specific aggregation (14), some contaminating connective tissue cells frequently overgrow the epithelial cells in serum-containing medium, especially on plastic dishes or attached collagen gels. No attempts have been made to identify the factors that inhibit fibroblast growth in our chemically defined medium.

Hormonal Requirement for Milk Protein Gene Expression

Because progesterone inhibits casein gene expression both in vivo (16) and in vitro (35), we removed this hormone from the medium in all the experiments designed to determine the hormonal requirements for milk protein gene expression. Hormonal requirement for milk protein gene expression is less stringent than for DNA synthesis and cell growth. We observed no difference in the amount of mRNA accumulated nor in the rate of protein synthesis and secretion by mammary cells when insulin, glucocorticoids, and PRL are present alone, as compared to medium supplemented with the hormones together with growth factors required for optimal growth. As already described in the accompanying paper (14), expression of milk protein genes is influenced by the shape of the mammary cells grown as multicellular structures. On a rigid substratum, the cells synthesize proteins, probably the ones required for cell survival, but not the milk proteins. The block in milk protein gene expression is not restricted to translational and posttranslational events, but also involves tbe transcription since no mRNA for casein accumulates in the attached mammary cells despite the presence of PRL stimulation. Lipogenesis, which is regulated by a PRL inducible acyl-transferase (4), is also inhibited. Release of cell constraints by freeing the collagen gels from the plastic wells leads to a rapid and drastic change in the geometry of the secretory cells. Concomitantly, in the absence of DNA synthesis and cell replication, cytodifferentiation occurs with the appearance of myoepithelial-like cells in the culture (14). A two- to threefold increase in overall protein synthetic activity accompanies these changes. In the absence of PRL, milk transferrin is synthesized and secreted, as well as the secretory component, a transmembrane protein acting as a receptor for polymeric immunoglobulins and mediating their transepithelial translocation (24) (Kraehenbuhl, unpublished observation). In contrast, the expression of caseins and α-lactalbumin genes requires the presence of PRL. Synthesis and secretion were dose-dependent with an optimal stimulation between 5×10^{-10} and 5×10^{-9} M, a concentration slightly higher than that required for the half-saturation of the cell surface receptors (38). Under optimal conditions, one secretory cell produces amounts of milk protein, which remain one or two orders of magnitude lower than those produced by a cell from a lactating gland in vivo, but are significantly higher than values reported in the literature (30, 31). At higher PRL concentrations, there is a drastic decrease in synthesis and secretion of caseins and α-lactalbumin. Such a dose-dependency is also observed at the transcriptional level (41), an observation which is interpreted by a down-regulation mechanism, whereby the availability of PRL receptors rapidly becomes too low to support the hormonal stimulation of milk protein gene expression (10). The PRL dependency of overall protein synthesis parallels that of casein and α-lactalbumin synthesis, an observation that is expected in view of the important contribution of these milk proteins (>70%) to total milk proteins (7).

When the number of secretory cells accumulating milk proteins is scored both on floating or in embedding gels, it appears that more cells store transferrin than caseins, although more caseins are secreted into the medium by cells grown on floating gels. Thus a third of the mammary cells are able to secrete about three times more casein than transferrin which is produced by two-thirds of the cells. In such calculations, one does not take into account the amount of transferrin released from the basolateral membrane of the cells grown on floating gels. Since casein and α-lactalbumin probes are available (39), and transferrin cDNA has recently been constructed, it will now be possible to study the metabolism of the mRNAs coding for these milk proteins and to determine whether differences exist in the half-lives of the various mRNA populations.

We are grateful to Dr. Richard Rodewald for helpful criticism and revision of the manuscript. We thank Mrs. Jacqueline Bonnard for typing the manuscript and Mr. Z. Freiwald for the graphic work.

This study was supported by grants from the Swiss National Science Foundation (3.672.0-80), the Swiss League Against Cancer (166 AK 80.1), and Nestle Nutrition.

Received for publication 30 August 1982, and in revised form 22 January 1983.

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