In Vitro Cell. Dev. Biol. 28A:625–634, September–October 1992

0883-8364/92 $01.50+0.00

HORMONAL INDUCTION OF FUNCTIONAL DIFFERENTIATION AND MAMMARY-DERIVED GROWTH INHIBITOR EXPRESSION IN CULTURED MOUSE MAMMARY GLAND EXPLANTS

B. BINAS, E. SPITZER, W. ZSCHIESCHE, B. ERDMANN, A. KURTZ, T. MÜLLER, C. NIEMANN, W. BLENAU, AND R. GROSSE[1]

Departments of Cellular Biochemistry (B. B., E. S., W. Z., A. K., T. M., C. N., W. B., R. G.) and Electron Microscopy (B. E.) of the Max-Delbrück Center for Molecular Medicine, Robert-Roessle-Str. 10, 0-1115 Berlin, Germany

(Received 3 February 1992; accepted 31 March 1992)

SUMMARY

A method for the cultivation of organ explants from abdominal mammary glands of virgin mice has been established. In a serum-free medium containing aldosterone, prolactin, insulin, and cortisol (APIH medium) mammary gland development was documented by lobuloalveolar morphogenesis. The hormonal requirements for in vitro expression of beta-casein and of the mammary-derived growth inhibitor (MDGI) were tested. To this end, a full length cDNA coding for mouse MDGI was prepared displaying strong homologies to a mouse heart fatty acid binding protein, which is also expressed in the mammary gland. MDGI and beta-casein transcripts were found to be absent in the mammary tissue from primed virgin mice, and were induced upon culture of mammary explants in the APIH medium. An immunohistochemical analysis with specific antibodies against MDGI and casein revealed a different pattern of expression for the two proteins. In the APIH medium, MDGI was expressed mainly in differentiating alveolar cells of the lobuloalveolar structures, whereas beta-casein was present in both ductules and alveoli. The relationship between functional differentiation and MDGI expression was further studied in explants from glands of late-pregnant mice. At this stage of development, MDGI is found both in ducts and in alveoli. If explants were cultured with epidermal growth factor (EGF) and insulin, the lobuloalveolar structure was still present, whereas MDGI disappeared. Reinduction of MDGI expression was achieved by subsequent PIH treatment. Independent on developmental stage, EGF strongly inhibits MDGI mRNA expression. It is concluded that MDGI-expression is associated with functional differentiation in the normal gland.

Key words: organ culture; mammary; explants; mouse; MDGI; differentiation.

INTRODUCTION

Development of the mammary gland is a complex multistage process (18,24,25). It begins in the embryo with the mammary anlagen which give rise to primary and secondary sprouts. Sparsely branching ducts invade the stroma at puberty, followed by the development of lobuloalveolar structures and functional differentiation, i.e. synthesis of milk proteins at pregnancy (2,18,24,25). By use of endocrine ablation (24,25), organ culture systems (2,10,17,19,30, 32,39), and various cell culture models (1,6,11,21,33,40) it has been documented that several hormones regulate this process [*see* (18) for a recent review]. The combined action of aldosterone, prolactin, insulin, and cortisol is sufficient to promote lobuloalveolar development and functional differentiation in whole organ cultures of mammary glands from sexually immature mice pretreated with estradiol and progesterone (2,32,34,38). In organ explant cultures from mammary glands of pregnant mice, functional differentiation can be maintained and further stimulated by prolactin, insulin, and cortisol (19,39). More recently, locally acting peptide growth factors have been implicated in both stimulation and inhibition of mammary epithelial cell proliferation and differentiation (6,9,12,22,32, 36). Little is known about the mechanisms regulating local expression of growth factors in the different cell types composing the mammary gland nor about their proliferative and/or differentiation modulating activities in the developing epithelium. In particular, this concerns the role growth inhibitors could fulfill during lobuloalveolar development. We have been studying the structure and function of the mammary-derived growth inhibitor (MDGI) which was purified from lactating bovine mammary gland and from milk fat globule membranes (4,5,15). We have recently cloned a cDNA encoding the bovine form of MDGI (20) and used it together with specific antibodies to analyze the expression of MDGI during different stages of development of the bovine mammary gland in vivo. However, there are no data available about in vitro MDGI expression and its dependence on endocrine hormones.

To address directly the question of a hormonal requirement for MDGI expression during ductal growth and lobuloalveolar differentiation, we have now employed a serum-free organ culture system with mouse mammary glands. Instead of using whole thoracic mammary glands as described before, we cultured explants from the abdominal glands of primed virgin mice. In this report, we describe that MDGI expression is induced in alveolar epithelial cells by a

[1] To whom correspondence should be addressed.

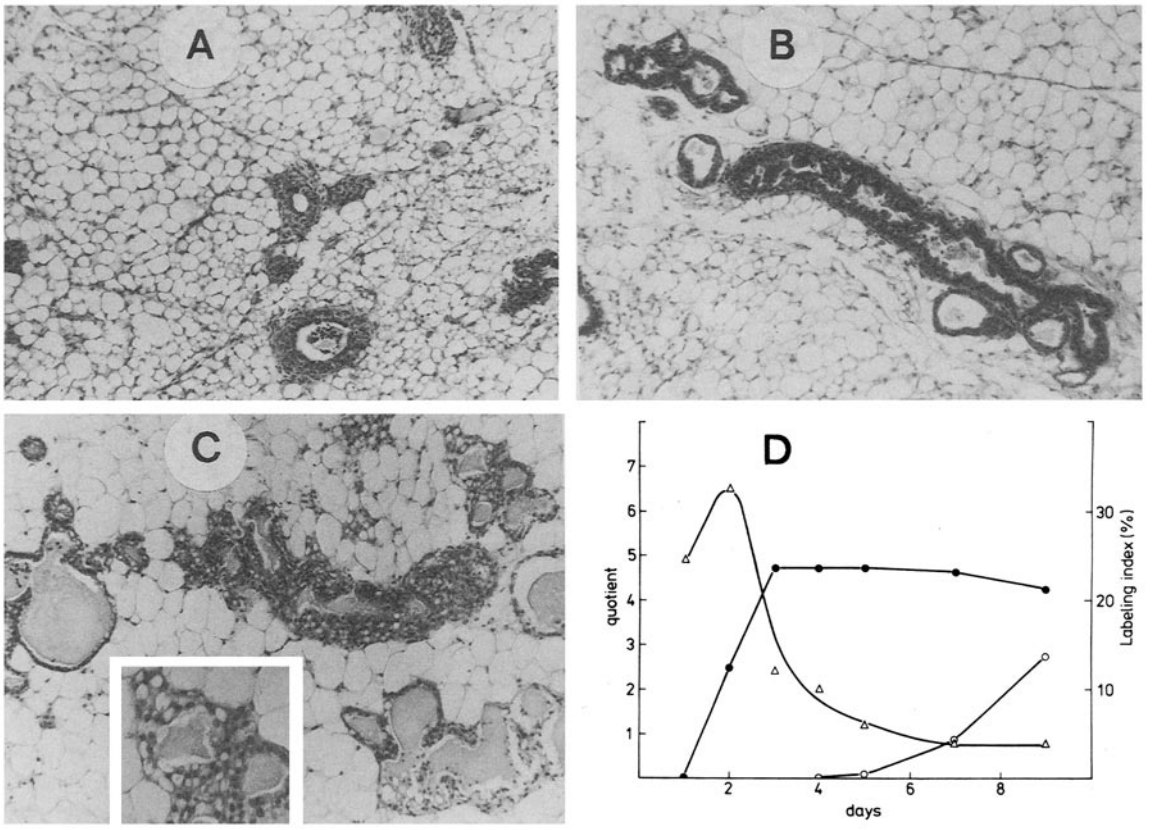

FIG. 1. Lobuloalveolar development in cultured mammary gland explants from primed virgin mice. Hematoxylin & eosin-stained paraffin sections of mammary explants before (*A*), and after Day 4 (*B*), or Day 9 (*C*) of organ culture in medium containing aldosterone, prolactin, insulin, and cortisol. Original magnifications, 80 (*A–C*) and 200 (*C, inset*). *Note* secretory droplets in alveolar cells and secretion into the lumen (*C, inset*). *D*, time course of growth parameters. For estimation of the LI of ductules (*triangles*), explants were pulsed with [^{3}H]thymidine for the final 4 h of culture. Quotients between the numbers of ductules to ducts (*closed circles*) and alveoli to ductules (*open circles*) were estimated morphometrically as outlined in Materials and Methods. Representative results are shown.

combination of hormones necessary to induce development of the mammary gland from primed virgin mice in vitro. In mammary explants from late-pregnant mice, MDGI expression is detected in ductal and alveolar cells and most likely closely associated with functional differentiation rather than with lobuloalveolar morphogenesis. We suggest a role of MDGI during differentiation associated with inhibition of growth of epithelial cells.

MATERIALS AND METHODS

Reagents. Ovine prolactin (no. L-6520, 31 IU/mg) and aldosterone (no. 12050) were from Sigma (Germany), and Serva (Germany), respectively. Cortisol (no. 24608) was obtained from Merck (Germany), and porcine insulin (40 IE/ml), stabilized with 1 mg/ml methylhydroxybenzoate, was from Berlin-Chemie, Germany. Epidermal growth factor (EGF) was prepared as described before (28). Medium 199 with Earle's salts and minimal essential medium (MEM) were purchased from SIFIN, Germany. Protein A-gold was prepared according to Slot and Geuze (29), and the IntenSE kit from Janssen (Belgium) was used for silver enhancement. (alpha-^{32}P)dATP and the DNA labeling kit were from Amersham (UK). [^{3}H]thymidine was the product of ZfK Rossendorf, Germany. All other chemicals were of highest analytical grade available.

Rabbit anti-mouse beta-casein antiserum (1) was kindly provided by Dr. R. Ball (Friedrich Miescher Institūt, Basel). The affinity-purified IgG fraction of a rabbit antiserum against bovine MDGI was described before (23). The 0.6 kb beta-casein cDNA in pUR51 (16) and the 0.58 kb beta-actin cDNA in pA3 (14) were provided by Drs. B. Groner (Friedrich Miescher Institūt, Basel) and R. Friis (Institūt für klinisch-experimentelle Tūmorforschūng, Universität, Bern), respectively. The 0.4 kb cDNA probe for mouse MDGI was prepared as described below.

Animals. Hormonally primed virgin mice, 5 wk old, and multiparous pregnant female mice of the Balb/c Han strain (bred in-house) were used. For priming (17), the virgin mice were injected subcutaneously for 9 consecutive days with 1 mg progesterone/1 μg estradiol (Jenapharm, Germany), dissolved in 50 μl of peanut oil.

Organ explant culture. The fourth (upper abdominal) mammary glands of hormonally primed virgin mice or the combined abdominal and thoracic glands of late-pregnant mice were removed, placed in HEPES-buffered MEM and sterilely cut into 1- to 2-mm pieces. Approximately 15 explants were obtained from one abdominal gland of a primed virgin mouse, and about 300 explants from the combined mammary glands of one pregnant mouse. The tissue pieces were then directly placed onto the untreated bottom of 25-cm^2 Falcon plastic flasks (no. 3013E) at a density of about 30 explants per flask. The explants adhered easily, and no cellular outgrowths were observed later during culture. After seeding the explants, MEM was replaced by 1.5 ml of Medium 199 with Earle's salts, gentamicin (50 μg/ml), and the hormone combinations. For culturing mammary gland explants from virgin mice, glucose concentration was raised to 11 m*M*. Hormone concentrations, if not otherwise stated, were 5 μg/ml for aldosterone, prolactin, and insulin. They were 1 μg/ml and 0.1 μg/ml for cortisol and EGF, respectively. The tissue was cultured under an atmosphere of 95% O_2:5% CO_2, with daily medium changes. Cultures were terminated by freezing the explants in liquid nitrogen (for extraction of RNA) or fixation in 4% formalin (for routine histology and immunohistochemistry). For autoradiography, explants were incubated with tritiated thymidine [^{3}H]TdR, 2

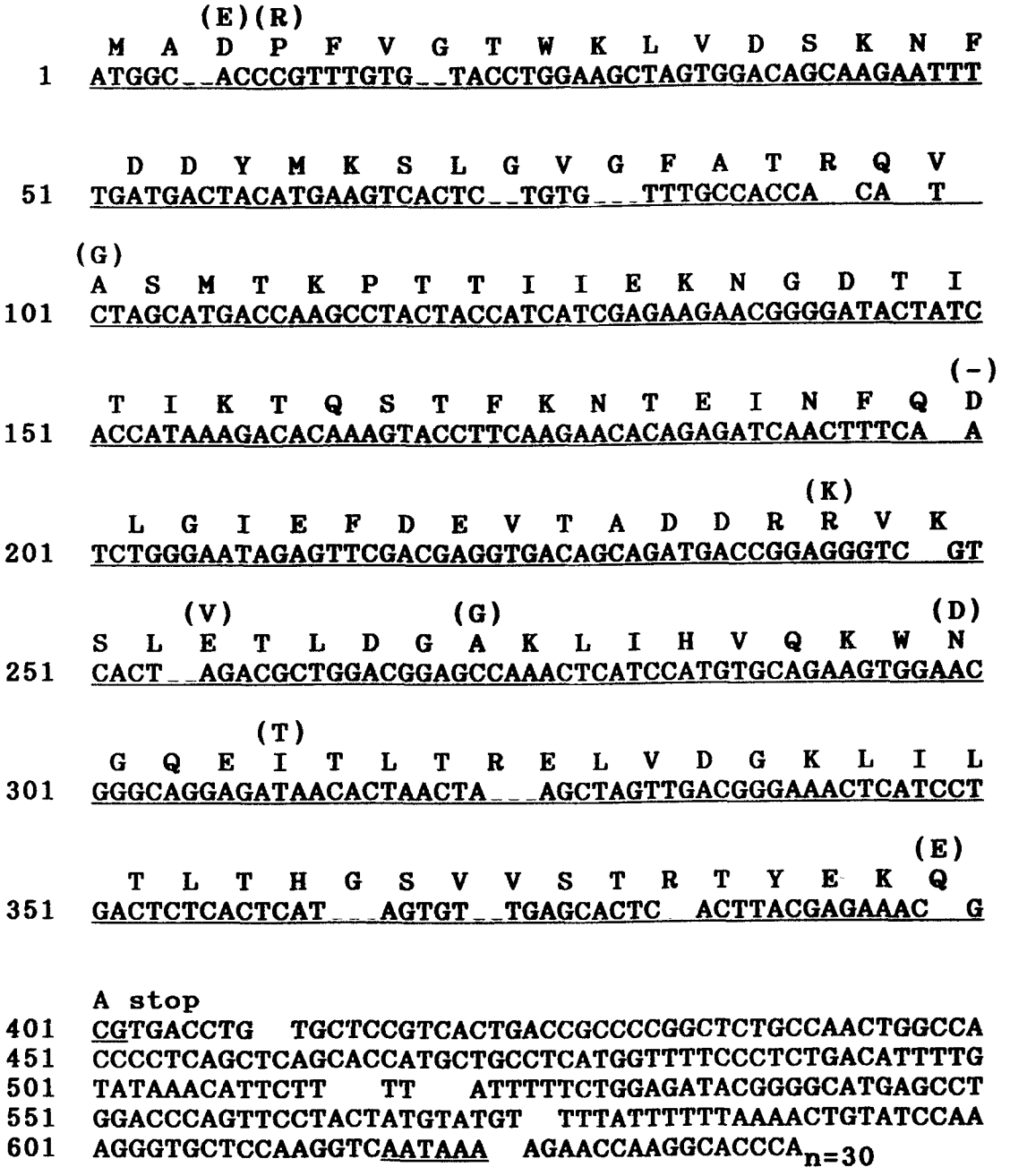

FIG. 2. Nucleotide and deduced amino acid sequence of mouse MDGI-cDNA. Residues that are *underlined* correspond to the amplified fragment used as hybridization probe in Northern blot experiments. The polyadenylation signal in the 3′ nontranslated region is also *underlined*. Amino acids that are not identical with those of the reported mouse heart FABP sequence (35) are shown in brackets.

μCi/ml, sp.act., 72 Ci/mmol; 1 Ci = 37 GBq) in fresh medium for the final 4 h of culture, and fixed thereafter in 4% formalin:0.5% trichloroacetic acid (TCA). All experiments outlined below and shown in Results have been performed at least in triplicates.

Histology and morphometry. Formalin-fixed explants were embedded in paraffin. Five-micrometer sections were cut, deparaffinized, and stained with hematoxylin and eosin. For quantitative evaluation of morphogenesis, numbers of ducts, ductules, and alveoli were counted in sections of at least five explants per experimental group at a magnification of 500:1. To compare the histologic pattern in different cultures, the quotients of the numbers of ductules per duct and alveoli per ductule were estimated in each of the sections. Histologic criteria employed were as follows: for ducts, an adjacent layer of connective tissue; for ductules, a linear coat of cubic epithelial cells without adjacent mesenchyme and signs of secretion; for alveoli, presence of secretory cells with vacuoles and luminal secretion. Only structures exhibiting lumina were counted. An experimental group comprised about 500 different structures for the morphometric analysis.

Thymidine autoradiography. Sections of explants fixed with 4% formalin:0.5% TCA were deparaffinized, incubated with an excess of cold thymidine overnight, and then dried and coated with K8 nuclear research emulsion (ORWO, Germany) at 1:2 dilution at 40° C. After exposure for 8 to 12 days at 4° C, sections were developed and counterstained with hematoxylin

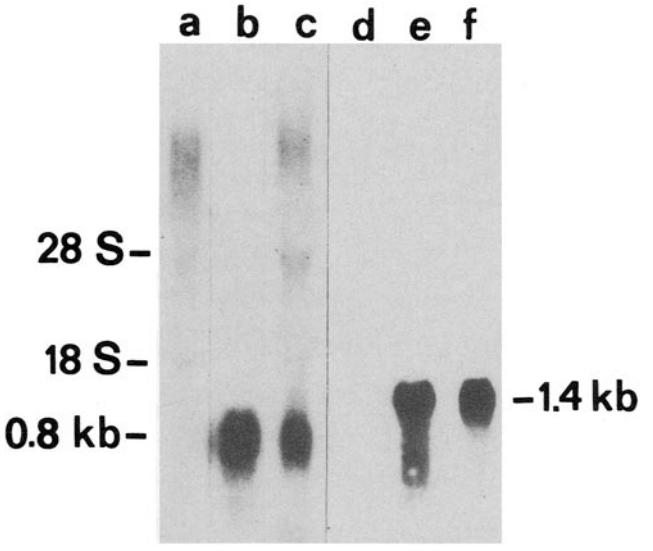

FIG. 3. Induction of MDGI-mRNA in mammary gland explants from primed virgin mice. Total RNA was extracted from the intact mammary glands of primed virgin (*lanes a,d*) or lactating (*lanes b,e*) mice, and from mammary gland explants after 9 days of culture with aldosterone, prolactin, insulin, and hydrocortisone (*lanes c,f*). The autoradiograph of the same Northern blot after hybridization with either the ^{32}P-labeled mouse MDGI-cDNA probe (*lanes a–c*) or beta-casein-cDNA probe (*lanes d–f*) is shown; 20 μg (*a,c,d,f*) or 10 μg (*b,e*) of total RNA were applied per lane. Typical results are shown.

& eosin or neutral fast red. At least 1000 nuclei were counted per experimental group and histologic structure. Labeling index (LI) was calculated as the percentage of nuclei with more than five silver grains per nucleus.

Immunohistochemistry. Sections of mammary explants were mounted on silane-activated glass slides and deparaffinized. They were treated subsequently with Lugol's iodine and sodium thiosulfate, and incubated with the specific antibodies at room temperature for 1 h. The rabbit anti-mouse beta-casein antiserum at a dilution of 1:3500 and the affinity-purified anti-MDGI-antibody at a concentration of 16 μg/ml were used in 20 m*M* Tris-HCl, pH 7.5, containing 500 m*M* NaCl, 0.1% bovine serum albumin, and 0.05% Tween 20. The sections were then washed with tris buffer/NaCl and incubated for 30 min with protein A-gold complexes (9 nm in diameter) in tris buffer/NaCl containing 0.1% bovine serum albumin. Silver enhancement was performed with the IntenSE kit for 8 min. Both antibodies have been extensively characterized earlier (1,23). Nonspecific staining was assessed by treating paraffin sections with antibodies preincubated with an excess of MDGI and by omission of the antibodies.

Cloning and sequencing of the mouse MDGI-cDNA. cDNA (10 ng) derived from the mammary glands of a pregnant mouse was used to amplify a specific mouse-MDGI fragment by the polymerase chain reaction (PCR) (26). Degenerated PCR-primers were deduced from the bovine MDGI amino acid sequence (5) at positions 1 to 7 (sense) and 126 to 133 (antisense), respectively. The amplified segment of 399 nucleotides was cloned into pUC 19, sequenced, and employed as a probe for RNA-blotting and for screening 2.5 × 10^5 plaques of a lambda gt10 cDNA library established from the mammary glands of a pregnant mouse. Positive clones were isolated, subcloned into pUC 19, and sequenced in both directions by the dideoxy chain-termination method (27).

Northern and slot blot hybridizations. Total RNA was extracted as described before (7). About 20 explants were pooled for one extraction. Each preparation was checked for integrity of ribosomal RNA. For Northern blot analysis, equal amounts of formaldehyde/formamide denatured total RNA were applied to 1% agarose gel and transferred to Hybond C-extra membranes (Amersham, UK) by capillary blotting (31). To ensure that equal amounts of RNA were loaded, the ethidium bromide-stained bands of 28S- and 18S-ribosomal RNAs in the agarose gels were photographed, and the photographs were scanned densitometrically. The blots were baked at 80° C for 2 h under vacuum, and prehybridized for 1 h at 60° C in 0.5 *M* sodium phosphate, pH 7.0, 1% sodium dodecyl sulfate (SDS)/1% bovine serum albumin (BSA)/1 m*M* EDTA (8). Under the same conditions, hybridization was performed for 15 to 20 h with 3 to 5 ng/ml of ^{32}P-labeled mouse MDGI-, beta-casein-, or beta-actin-cDNA-probes. Linearized plasmids were labeled to a specific activity of 5 × 10^8 to 2 × 10^9 cpm/μg by the random hexanucleotide primed synthesis method (13). The relative intensities of hybridization signals were estimated using a Shimadzu scanning densitometer. Hybridization signals of slot blots and Northern blots were, respectively, normalized to the corresponding beta-actin signal or the amount of 28S ribosomal RNA determined as described above.

RESULTS

Induction of morphogenesis in explants from virgin mice. Explants taken from the abdominal glands of primed virgin mice have not been cultured in serum-free hormonally defined media before. We therefore first tested the responsiveness of explants to hormonal treatment. The mammary gland of 4-wk-old virgin Balb/c mice consisted of a system of sparsely branching ducts embedded in adipose tissue (Fig. 1 *A*). Upon cultivation with aldosterone, prolactin, insulin, and cortisol (APIH medium) for 4 days, ductules were sprouting from larger ducts into the fat pad (Fig. 1 *B*). They formed lobules that eventually differentiated into lobuloalveolar structures (Fig. 1 *C*), exhibiting cubic epithelial cells with fat vacuoles and varying amounts of eosinophilic secretory material in their lumina (Fig. 1 *C, inset*). A more detailed analysis of this process was performed by use of thymidine autoradiography and morphometry. Representative results are illustrated in Fig. 1 *D*. As demonstrated, the first 2 to 3 days of culture were characterized by a wave of DNA synthesis in the emerging ductules declining to a low level by Day 5. Ductal DNA synthesis was always lower than that of ductules (not shown). It began with a labeling index of about 13% and approached the LI for alveoli at the end of culture. Figure 1 *D* also showed that the ratio between ductules and ducts increased during the proliferative phase, reaching some constant level just after the wave of DNA synthesis had been accomplished. Beginning with Day 5, lobuloalveolar differentiation as expressed by the ratio between alveoli and ductules became apparent and progressed up to the end of culture at Day 9.

Cloning of the mouse MDGI-cDNA. To estimate MDGI mRNA levels, a specific mouse MDGI-cDNA was prepared from the cDNA synthesized from RNA of pregnant mouse mammary gland by the polymerase chain reaction. The PCR-fragment of murine MDGI-cDNA was then used to screen a lambda gt 10 cDNA library which was derived from the mammary gland of pregnant mouse. Nucleotide analysis of cDNA clones revealed existence of two closely related clones: one cDNA clone turned out to be identical with the mouse cardiac FABP (35), the second clone differed from this in 10 positions of the predicted amino acid sequence and is here designated as the murine form of MDGI (Fig. 2). At position 67, the mouse MDGI-cDNA codes for an additional aspartic acid residue which is not present in heart FABP.

Hormonal induction of MDGI expression in explants of virgin mice. We know that in the bovine mammary gland the MDGI expression increases during pregnancy and lactation (20). It was therefore of interest to investigate whether MDGI-expression was under hormonal control and associated with lobuloalveolar morphogenesis. To this end, MDGI transcription was measured in parallel

to the beta-casein expression. First, the specific mouse MDGI-cDNA probe was used to follow MDGI transcription in the developing mammary gland in vitro. In mammary tissue of primed virgin mice, MDGI was not detectable (Fig. 3, *lane a*). In mammary glands of lactating mice, the 0.8 kb transcript was clearly present (Fig. 3, *lane b*). A transcript of the same size could be induced in mammary gland explants from primed virgin mice upon cultivation in the APIH medium (Fig. 3, *lane C*). An additional band of unknown origin was seen frequently in tissues from virgin gland. The same blots were probed for beta-casein (Fig. 3, *lanes d–f*). The hormonal cocktail caused functional differentiation of mammary glands as indicated by APIH-dependent induction of beta-casein gene transcription (Fig. 3, *lane f*) if compared to the controls (*lanes d,e*).

We next addressed the question, Is the MDGI transcription accompanied by MDGI protein expression in mammary gland explants from virgin mice when cultured in APIH medium? To this end, paraffin-embedded tissue sections were analyzed by using an anti-MDGI antibody (Fig. 4). The antibody specifically detects the 14.5 kDa bovine MDGI protein in Western blots (23). The same result was observed with extracts from mouse mammary gland explants (not shown). As depicted in Fig. 4 *A*, no MDGI was detectable in mammary glands of primed virgin mice before the onset of culture. After 3 days in culture, positive staining was observed over emerging lobuloalveoli (Fig. 4 *B*). In 9-day explants, the secretory alveolar cells, preferentially those endowed with fat globules, exhibited strong immunostaining (Fig. 4 *C*). In contrast, both ductules and ducts as well as the glandular lumina were negative. This finding is clearly different from that for casein expression, which was found to occur after 9 days in culture both in ductular and alveolar epithelial cells after consecutive sections were immunostained with a specific anti-beta-casein antiserum (Fig. 4 *F*). It is an advantage of using the virgin mammary gland that at the beginning of culture the explants do not produce detectable amounts of MDGI and beta-casein (Fig. 4 *A,D*). After 3 days in culture, casein expression is induced by APIH (Fig. 4 *E*). To prove whether the medium contains beta-casein, conditioned medium proteins of cultures maintained in the APIH medium were concentrated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), electroblotted onto nitrocellulose and analyzed for beta-casein with the anti-beta-casein antiserum (Fig. 4 *F, inset lower right*). As shown, the explants are capable of releasing the milk protein.

Mammary-derived growth inhibitor expression is associated with functional differentiation. The data show that both lobuloalveolar development and functional differentiation are associated with MDGI expression. To get further insight into the problem whether MDGI expression is coupled to lobuloalveolar morphology or rather associated with functional differentiation, we have taken advantage of studies with mammary glands from late-pregnant mice. It can be concluded from earlier studies that in the absence of prolactin and cortisol, the addition of EGF supports maintenance of lobuloalveolar morphology under conditions when functional differentiation, i.e. casein expression, is prevented (39). Therefore, for testing MDGI expression in explants not expressing beta-casein, mammary glands from late pregnant mice (Fig. 5 *A*) were cultured for 5 days with EGF and insulin (EI medium). In dependence on the EI-medium epithelial cells lose their secretory appearance (Fig. 5 *B*); however, glandular tissue still keeps the lobuloalveolar morphology typically for the differentiated state maintained by prolactin, insulin and cortisol (PIH) (Fig. 5 *C*). In glands taken and cultured from pregnant mice, MDGI was immunochemically detected in alveoli and less abundant in ducts and ductules (Fig. 5 *D,F*). There was also some staining in nonvacuolized ducts. Both findings differ from those described before for glands of virgin mouse.

The shift to an EI medium was accompanied by a strong suppression of MDGI expression as deduced from the immunohistochemical analysis (Fig. 5 *E*). Under these conditions, explants otherwise keep morphology of the differentiated phenotype (*compare* Fig. 5 *B* and *C*). Upon culture in the PIH medium, expression of MDGI remains qualitatively unchanged (Fig. 5 *F*) if compared to the original gland (Fig. 5 *D*). These findings indicate that MDGI is not just required for maintaining morphologic structures during differentiation. In keeping with the immunohistochemical data, MDGI and beta-casein transcription were strongly inhibited by omission of prolactin and cortisol (Table 1). The data also indicate that even at optimal prolactin concentrations the MDGI mRNA level decreased during time of culture.

To further test whether MDGI synthesis is required for maintenance of morphology of differentiated alveoli, or associated with functional differentiation, we maintained cultures first under de-differentiating conditions and subsequently exposed them to lactogenic hormones (Fig. 6). To this end, explants from late-pregnant mice were first treated with EI medium for 1 day. As shown during that time the MDGI mRNA level declined to background levels, whereas for casein some residual expression was estimated. Then the EI medium was changed for the PIH medium, the explants were cultured for another 2 days, and again analyzed for MDGI and casein expression. As shown, prolactin and cortisol re-induced MDGI and casein expressions, although they were more effective in the case of beta-casein. Finally, we asked whether EGF, known for its antagonistic effects on MDGI-dependent growth inhibition (15) and for its dedifferentiating action in mouse mammary gland (37), could suppress MDGI expression in vitro. As shown in Table 2, independently of whether immature glands of virgin mice or fully differentiated glands were taken into culture, a strong EGF-dependent inhibition of MDGI mRNA expression was observed.

Discussion

The culture model we presented here is suitable to study developmental processes occurring in the virgin abdominal mammary gland under hormonal control in a serum-free medium. So far, this has been achieved with organ cultures of the whole thoracic mammary gland from primed virgin mice (2,17,32,34,38). It seems to be an advantage of the explant culture that, due to the large surface, secretory proteins are easily detected in the conditioned media. The explant technique can be used to scale-up the production of conditioned media for preparative purposes by culturing explants in a perfusion system under continuous medium supply (B. Binas et al., manuscript in preparation).

We have shown here that in the presence of prolactin, cortisol, aldosterone, and insulin the ductal epithelium of abdominal mammary gland explants from primed virgin mice undergo lobuloalveolar development and functional differentiation and that these processes are associated with onset of MDGI expression. We knew from experiments that the expression of MDGI cannot be easily achieved in monolayer cultures. For example, contrary to the induction of casein expression in mouse HC11 cells (22) or in mouse

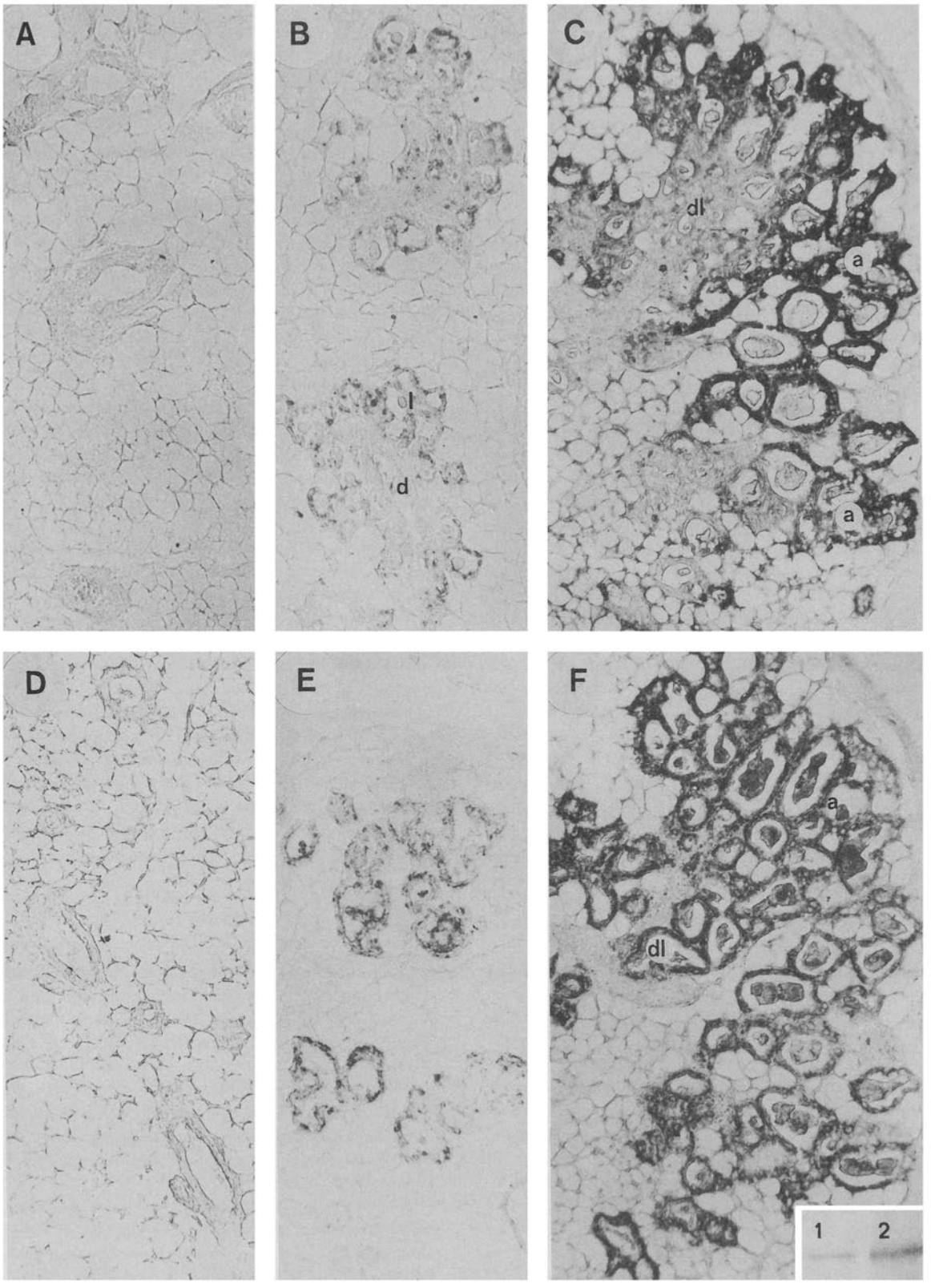

FIG. 4. Induction and immunohistochemical distribution of MDGI in cultured mammary gland explants from primed virgin mice. Histologic sections of mammary explants from virgin mice at the beginning (*A*), at Day 3 (*B*), or Day 9 (*C*) of culture with APIH medium were incubated with anti-MDGI antibodies and the immunologic reaction was visualized by the immunogold/silver enhancement procedure as described in Materials and Methods. Original magnification, 80. *Note* that after 9 days in culture preferentially alveoli are

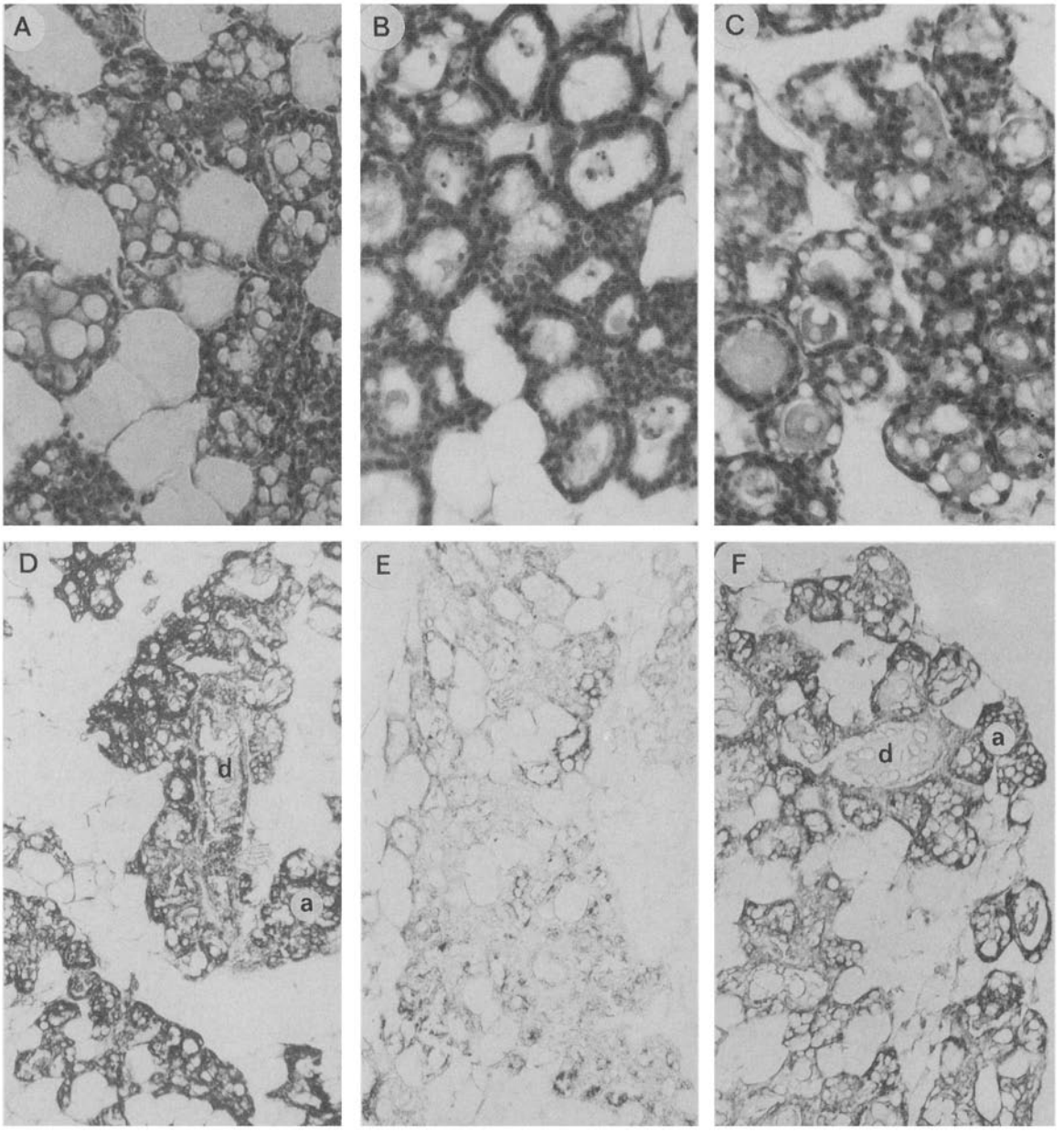

FIG. 5. Relationship between MDGI expression and functional differentiation in mammary gland explants from pregnant mice. Hematoxylin & eosin-stained sections (*A–C*) (original magnification, 200) and paraffin sections immunostained for MDGI detection (*D–F*) (original magnification, 80) are shown. *A*, mammary gland before culture; *B*, tissue after 5 days in culture with EGF and insulin; *C*, after 5 days in culture with prolactin, cortisol, and insulin; *D*, MDGI expression in the mammary gland from pregnant mouse; *E*, after 5 days in culture with EGF and insulin; *F*, after 5 days in culture with prolactin, cortisol, and insulin. *Note* MDGI immunostaining in alveoli and to a less extent in ductal structures (*E*), maintenance of the secretory state after cultivation in PIH-medium (*C*), and nonsecretory alveolar epithelium upon cultivation with EGF (*B*). *Note* disappearance of MDGI expression in lobuloalveolar structures preserved by EGF and insulin (*E*). Results of a representative experiment are shown.

immunostained, whereas ducts and ductules only occasionally were positive. Results typical of several independent experiments are shown. In parallel, sections from virgin mice at the beginning (*D*), at Day 3 (*E*), and Day 9 (*F*) in culture with APIH were analyzed with a specific anti-beta-casein antiserum. *C* and *F*, represent consecutive sections to demonstrate the different pattern of staining for MDGI and casein, respectively. *Note* strong staining for casein of alveolar and ductular epithelium in explants cultured for 9 days. *dl*, ductulus; *a*, alveolus; *d*, duct; *l*, lobulus. *Inset* to *F*: Detection of secreted beta-casein in the culture medium. *1*, mouse milk standard, *2*, conditioned medium. The TCA-precipitated proteins of 2.5 ml of conditioned medium were subjected to SDS-PAGE and Western blotting, and the blot was incubated with anti-beta-casein antiserum and developed with alkaline phosphatase-conjugated secondary antibody. Representative results are shown.

TABLE 1

HORMONAL REQUIREMENT FOR MAINTENANCE OF MDGI-mRNA EXPRESSION[a]

	Beta-Casein-mRNA	MDGI-mRNA
Day 0	100	100
Day 4, PIH	187 ± 90	27 ± 25
Day 4, EI	0	0
	(n = 3)	(n = 3)

[a] Total RNA was extracted from mammary gland explants of late-pregnant mice before (Day 0) or after 4 days of culture with PIH. Alternatively, both prolactin and cortisol were omitted and replaced by 0.1 μg/ml EGF (EI). The Northern blot was hybridized consecutively with the ^{32}P-labeled mouse MDGI-cDNA and beta-casein-cDNA probes; 20 μg of RNA were applied per lane. Hybridization signals were normalized to the content of 28 S ribosomal RNA as described in Materials and Methods. Values are expressed as percent of the RNA level in tissue before taken into culture. Standard deviations were calculated from data of three independent experiments. The response to prolactin of casein and MDGI expression varied in the experiments. However, MDGI-expression was consistently lower compared with casein at all prolactin concentrations (0.02, 0.05, 0.5, and 5 μg/ml) tested.

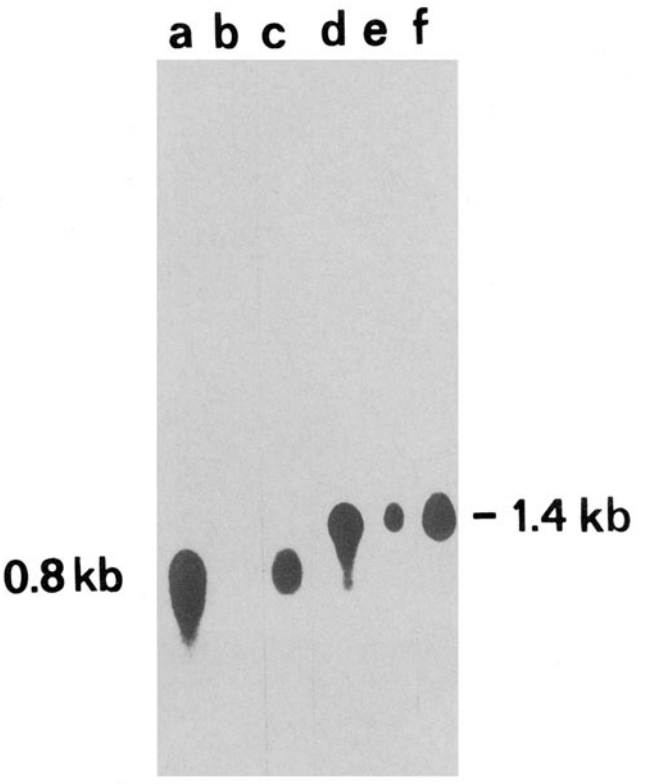

FIG. 6. Reinduction of MDGI mRNA in cultured mammary gland explants from pregnant mice. Explants from pregnant mice were cultured for 24 h with EI medium, washed 3 times with basal medium, and then shifted to the PIH medium for another 48-h culture period. Samples were withdrawn for extraction of total RNA before culture (*lanes a,d*) and after culture in the EI (*lanes b,e*) or PIH medium (*lanes c,f*). Same Northern blots were hybridized either with the ^{32}P-labeled mouse MDGI-cDNA (*lanes a–c*) or beta-casein-cDNA (*lanes d–f*); 10 μg of total RNA were applied per lane. Results of a representative experiment are shown.

mammary organoids, we were not able to trigger MDGI synthesis by dexamethasone, prolactin, and insulin in these cultures (M. Mieth, unpublished data). Also, the mRNA level for MDGI was estimated to be less than 0.01% of the total RNA content in murine and human epithelial cells maintained in monolayer cultures (23).

Concerning the protein expression in the mammary gland from virgin mice cultured with APIH, MDGI expression differs from that of casein. MDGI expression seems to be restricted to the differentiating alveoli in cultures from primed virgin mice. This is not the case for casein, which was found in ductules and alveoli. The pattern of MDGI immunostaining thus resembles the MDGI-mRNA expression we described earlier for the mammary gland of pregnant cows by use of an in situ hybridization analysis (20). In organ explants from late-pregnant mice the MDGI expression was no longer restricted to alveoli, being now similar to that of casein. Again, this is in keeping with the data of the in situ analysis (20).

The functional consequence of the different patterns of MDGI expression and its possible relationship with enhanced lipid synthesis during terminal differentiation requires further investigation. Recently the cDNA for bovine MDGI has been cloned from a library that was derived from terminally differentiated bovine mammary gland (20). This cDNA-deduced MDGI sequence is identical with that reported for the bovine heart FABP (3), but differed from the protein-derived amino acid sequence in six positions. The authors could not determine whether different forms of MDGI exist in the mammary gland. In this regard, our present data on mouse mammary gland suggest the existence of MDGI isoforms. Again, one of them is identical with the cardiac type of FABP (35). The functional meaning of expression of MDGI-related proteins in heart, brain, mammary gland, or kidney is unknown (15). Recently a selenium-binding protein has been purified from mouse mammary gland, which is close but not identical with MDGI or cardiac FABP (D. Medina, personal communication). Thus, a family of proteins fulfilling different functions seems to emerge. At present no probes are available to distinguish between them.

Mammary-derived growth inhibitor expression can be induced by a hormone combination known to stimulate morphogenesis and casein expression. To inhibit MDGI mRNA expression, it was sufficient to culture explants from pregnant mice with EGF and insulin for 24 h. Under these conditions the lobuloalveolar structure of differentiated mammary glands did not change. The subsequent

TABLE 2

SUPPRESSION OF MDGI EXPRESSION BY EGF[a]

	mRNA Content	
Physiological State	Virgin	Pregnant
Control	100	100
+EGF	18 ± 2	30 ± 16
	(n = 2)	(n = 13)

[a] Culture conditions as described in Methods, i.e. in APIH medium for 5 days and in PIH medium for 2 days for mammary glands from virgin and pregnant mice, respectively. All experiments were performed at least in triplicate. Content of mRNA was estimated from Northern blots as described. EGF did not significantly affect casein expression tested in parallel experiments.

treatment with lactogenic hormones re-induced MDGI expression in preformed alveoli. In addition, EGF effectively inhibits MDGI-mRNA expression under conditions of ongoing morphogenesis in the cultured mammary gland of virgin mice, or in terminally differentiated cells during late pregnancy. This indicates that MDGI is not simply linked to a morphologic phenotype of the differentiated secretory epithelial cell and that an EGF-dependent mechanism of regulation of MDGI expression could be involved. The data suggest a close relationship between MDGI and hormonally controlled functional differentiation. It remains to be elucidated whether prolactin alone or in combination with cortisol and other hormones induces MDGI gene transcription and expression. So far, we cannot answer the question whether MDGI expression is controlled exclusively on the transcriptional level or also posttranscriptionally by mechanisms influencing stability or half-life time of its mRNA. The apparent close parallel between expression of MDGI and of its mRNA during development of the mammary gland from cow (20) and mouse suggests that transcriptional mechanisms are important. In summary, the MDGI expression in mammary epithelial cells is under the control of mammogenic hormones and closely associated with functional differentiation. We currently employ the culture system to address the questions how MDGI and synthetic MDGI-peptides directly and in combination with prolactin and EGF control proliferation and differentiation of mammary epithelial cells in the developing mouse mammary gland.

Acknowledgements

We thank R. Ball, B. Groner, and R. Friis for providing the anti-mouse-beta-casein antiserum, the mouse beta-casein cDNA, and the beta-actin-cDNA, respectively. The technical assistance of A. Jaetsch, K. Feller, E. Kotitschke, S. Nowak, and A. Dell'Oro is gratefully acknowledged. We are grateful to I. Wiznerowicz for excellent typing of the manuscript.

References

1. Ball, R. K.; Friis, R. R.; Schoenenberger, C. A., et al. Prolactin regulation of β-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J. 7:2089–2095; 1988.
2. Banerjee, M. R.; Antoniou, M. Steroid and polypeptide hormone interaction in milk-protein gene expression. In: Litwack, G., ed. Biochemical actions of hormones, vol. 12. New York: Academic Press; 1985:237–289.
3. Billich, S.; Wissel, T.; Kratzin, H., et al. Cloning of a full-length complementary DNA for fatty-acid-binding protein from bovine heart. Eur. J. Biochem. 175:549–556; 1988.
4. Boehmer, F.-D.; Lehmann, W.; Noll, F., et al. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. Biochem. Biophys. Acta 846:145–154; 1985.
5. Boehmer, F.-D.; Kraft, R.; Otto, A., et al. Identification of a polypeptide growth inhibitor from bovine mammary gland. J. Biol. Chem. 262:15137–15143; 1987.
6. Chen, L.-H.; Bissell, M. J. A novel mechanism for whey acidic protein gene expression. Cell Regul. 1:45–54; 1989.
7. Chomczynsky, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform-extraction. Anal. Biochem. 162:156–159; 1987.
8. Church, G. M.; Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991–1995; 1984.
9. Coleman, S.; Daniel, C. W. Inhibition of mouse mammary ductal morphogenesis and down-regulation of the EGF-receptor by epidermal growth factor. Dev. Biol. 137:425–433; 1990.
10. Du Bois, M.; Elias, J. J. Subpopulations of cells in immature mouse mammary gland as detected by proliferative responses to hormones in organ culture. Dev. Biol. 106:70–75; 1984.
11. Emerman, J. T.; Pitelka, D. R. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro 13:316–328; 1977.
12. Ervin, P. R., Jr.; Kaminski, M. S.; Cody, R. C., et al. Production of mammastatin, a tissue specific growth inhibitor, by normal human mammary cells. Science 244:1585–1587; 1989.
13. Feinberg, A. P.; Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266–267; 1984.
14. Ginzberg, J.; de Baetselier, A.; Walker, M. D., et al. Brain tubulin and actin cDNA sequences: isolation of recombinant plasmids. Nucleic Acids Res. 8:3553–3564; 1980.
15. Grosse, R.; Langen, P. Mammary-derived growth inhibitor. In: Born, G. V. R.; Cuatrecasas, P.; Herken, H., et al. Handbook of experimental pharmacology, vol. 95/II. Heidelberg: Springer-Verlag; 1990:249–265.
16. Hennighausen, L.; Sippel, A. E. Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. Eur. J. Biochem. 125:131–141; 1982.
17. Ichinose, R. R.; Nandi, S. Influence of hormones on lobuloalveolar differentiation of mouse mammary glands in vitro. J. Endocrinol. 35:331–340; 1966.
18. Imagawa, W.; Bandyopadhyay, G. K.; Nandi, S. Regulation of mammary epithelial cell growth in mice and rats. Endocr. Rev. 11:494–523; 1990.
19. Juergens, W. G.; Stockdale, F. E.; Topper, Y. J., et al. Hormone-dependent differentiation of mammary gland in vitro. Proc. Natl. Acad. Sci. USA 54:629–634; 1965.
20. Kurtz, A.; Vogel, F.; Funa, K., et al. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. J. Cell Biol. 11:1779–1789; 1990.
21. Levay-Young, B. K.; Hamamoto, S.; Imagawa, W., et al. Casein accumulation in mouse mammary epithelial cells after growth stimulated by different hormonal and nonhormonal agents. Endocrinology 126:1173–1182; 1990.
22. Mieth, M.; Boehmer, F.-D.; Ball, R., et al. Transforming growth factor-β inhibits lactogenic hormone induction of β-casein-expression in HC11 mouse mammary epithelial cells. Growth Factors 4:9–15; 1990.
23. Mueller, T.; Kurtz, A.; Vogel, F., et al. A mammary-derived growth inhibitor (MDGI) related 70 kDa antigen identified in nuclei of mammary epithelial cells. J. Cell. Physiol. 138:415–423; 1989.
24. Nandi, S. Endocrine control of mammary gland development and function in the C3H/He Crgl mouse. JNCI 21:1039–1063; 1958.
25. Nandi, S. Hormonal control of mammogenesis and lactogenesis in the C3H/He Crgl mouse. Univ. Calif. Publ. Zool. 65:1–128; 1959.
26. Saiki, R. K.; Gelfaud, D. H.; Stoffel, S., et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA-polymerase. Science 239:487–491; 1988.
27. Sanger, F.; Nicklen, S.; Coulson, A. R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467; 1977.
28. Savage, C. R., Jr.; Cohen, S. Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. 247:7609–7611; 1972.
29. Slot, J. W.; Geuze, H. J. A new method of preparing gold probes for multiple-labelling studies. Eur. J. Cell Biol. 38:87–93; 1985.
30. Smith, G. H. Functional differentiation of virgin mouse mammary epithelium in explant culture is dependent upon extracellular proline. J. Cell. Physiol. 131:190–199; 1987.
31. Thomas, P. S. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77:5201–5205; 1980.
32. Tonelli, Q. J.; Sorof, S. Epidermal growth factor requirement for development of cultured mammary gland. Nature 285:250–252; 1980.
33. Tonelli, Q. J.; Sorof, S. Induction of biochemical differentiation in

three-dimensional collagen cultures of mammary epithelial cells from virgin mice. Differentiation 22:195–200; 1982.
34. Tonelli, Q. J.; Sorof, S. Expression of a phenotype of normal differentiation in cultured mammary glands is promoted by epidermal growth factor and blocked by cyclic adenine nucleotide and prostaglandins. Differentiation 20:253–259; 1981.
35. Tweedie, S.; Edwards, Y. cDNA sequence for mouse-heart fatty acid binding protein, H-FABP. Nucleic Acids Res. 17:4374; 1989.
36. Vonderhaar, B. K. Local effects of EGF, alpha TGF and EGF-like growth factors on lobulo-alveolar development of the mouse mammary gland in vivo. J. Cell. Physiol. 132:581–584; 1987.
37. Vonderhaar, B. K.; Nakhasi, H. C. Bifunctional activity of epidermal growth factor on alpha- and kappa-casein gene expression in rodent mammary glands in vitro. Endocrinology 119:1178–1184; 1986.
38. Wood, B. G.; Washburn, L. L.; Mukherjee, A. S., et al. Hormonal regulation of lobulo-alveolar growth, functional differentiation and regression of whole mouse mammary gland in organ culture. J. Endocrinol. 65:1–6; 1975.
39. Yoshimura, M.; Oka, T. Hormonal induction of β-casein gene expression: requirement of ongoing protein synthesis for transcription. Endocrinology 126:427–433; 1990.
40. Yoshimura, M.; Oka, T. Transfection of β-casein chimeric gene and hormonal induction of its expression in primary murine mammary epithelial cells. Proc. Natl. Acad. Sci. USA 87:3670–3674; 1990.