Developmental Regulation of Mammary-derived Growth Inhibitor Expression in Bovine Mammary Tissue

Andreas Kurtz,* Frank Vogel,* Keiko Funa,‡ Carl-Henrik Heldin,‡ and Richard Grosse*

*Departments of Cellular Biochemistry and Electron Microscopy, Academy of Science of the German Democratic Republic, Central Institute of Molecular Biology, Berlin-Buch, German Democratic Republic; and ‡Ludwig Institute for Cancer Research, Uppsala, Sweden

Abstract. The cDNA for a previously described growth inhibitor, designated as mammary-derived growth inhibitor (MDGI) (Grosse, R., and P. Langen. 1989. *In* Handbook of Experimental Pharmacology. In press) has been cloned from a plasmid library which was derived from terminally differentiated bovine mammary gland.

Sequencing of the cDNA showed an open reading frame coding for a protein of 133 amino acids. In six positions differences were found between the sequence determined from the cDNA and that determined previously by amino acid sequence analysis. Northern blot analysis revealed abundant MDGI mRNA in the terminally differentiated mammary gland, whereas in virgin gland, liver or pancreas transcripts were not expressed. By use of in situ hybridization technique transcription of MDGI in the developing bovine mammary gland was analyzed.

Increasing amounts of MDGI mRNA were detected in the epithelial cells of embryonic mammary rudiment, in the epithelium of developing lobules and in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands. There was a geographical gradient of MDGI mRNA concentration in bovine mammary gland reaching a maximum in the proximal parts of the tissue. An immunohistochemical analysis with different polyclonal and peptide directed antibodies against MDGI confirmed the in situ hybridization data with respect to the tissue-specific and differentiation-dependent MDGI expression in bovine mammary gland. The results suggest a close relationship between MDGI transcription and developmental processes in the normal bovine mammary gland.

DEVELOPMENT and differentiation of mammary tissue is driven by a combination of hormonal and local controls (17, 22, 30, 57, 58). In vitro studies with organ cultures (19, 45, 56, 62) and with mammary epithelial cells growing either within collagen gels (27, 49) or as monolayers (2, 31) identified the importance of steroid hormones and polypeptide growth factors for glandular morphogenesis and differentiation.

In virgin animals mammary gland morphogenesis stops at an immature state characterized by preformed branching ducts terminated by stem cell populations with the property to form during pregnancy lobuloalveolar epithelial and myoepithelial cells (18, 27). During pregnancy the ductal tree undergoes growth and differentiation culminating in a functionally specialized lobuloalveolar gland capable of milk synthesis and secretion. Little is known about the regional and developmental expression of locally acting growth factors and growth inhibitors in this periodically regenerating epithelium. Knowledge of the expressional pattern of growth factors potentially involved in mammary development and differentiation may be useful for better understanding of normal and neoplastic growth.

Among the known polypeptide growth factors most studies have been devoted to EGF for mammary development. Although a local synthesis and expression in the mammary gland has not been shown the mitogenic activity of EGF-like factors seems necessary for growth and development of the epithelium (22, 32, 55, 56, 59).

The existence of growth inhibitory polypeptides controlling growth, differentiation, and regression of the epithelium in vivo has been postulated (22, 24, 39). For transforming growth factor beta, which inhibits proliferation and lobuloalveolar outgrowth in the mammary gland of virgin mice, a similar role was discussed (53).

More recently a protein called mammastatin has been purified from conditioned media of normal mammary epithelial cells. This polypeptide blocks DNA synthesis in cultured normal and transformed mammary epithelial cells and is secreted by the same cells (20).

Another protein involved in growth inhibition has been described by us (8). A 13-kD growth inhibitor, designated mammary-derived growth inhibitor (MDGI)[1] has been purified

1. *Abbreviations used in this paper*: FABP, fatty acid binding protein; MDGI, mammary-derived growth inhibitor; PCR, polymerase chain reaction.

from the lactating bovine mammary gland and from milk fat globule membranes (11). MDGI reversibly inhibits the proliferation of several normal and transformed mammary epithelial cells in vitro (7, 8, 24). The inhibitor shares extensive sequence similarity to a family of proteins with the common feature to bind hydrophobic ligands such as retinoic acid or fatty acids (6, 8).

To gain further insights into the spatial and temporal MDGI expression during mammary gland development we have used a cloned cDNA and different anti-MDGI antibodies to investigate the tissue specific and cellular distribution of this growth inhibitor. The results clearly show that MDGI is transcribed in mammary epithelial cells of developing lobuloalveolar structures reaching abundance in proximal parts of the terminally differentiated gland. Striking RNA concentrations were found in the terminal parts of differentiated ducts. The level of MDGI mRNA parallels the appearance of the protein it encodes thus suggesting a transcriptional control of MDGI expression during mammary development.

Materials and Methods

Synthesis of the Bovine Mammary Gland cDNA Library

RNA was isolated from lactating bovine mammary gland according to Chomczynsky and Sacchi (14). Poly (A)-containing mRNA was prepared by chromatography on Poly (U)-Sepharose (Pharmacia, Uppsala, Sweden) and complementary DNA was synthesized according to Gubler and Hoffman (25) with the modifications introduced by the Amersham Corp. (Arlington Heights, IL) cDNA synthesis kit. The cDNA was ligated into the Hinc II site of pUC 19 (43) and transformed into *Escherichia coli* DH5a yielding a library of 3×10^5 independent clones.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed as described (48). 10 ng of the bovine mammary gland double-stranded cDNA or 2 μg genomic DNA in 0.1 ml PCR-buffer consisting of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatine, 4 μM each of the different oligonucleotide PCR primers (A_1, A_2, B_1, and B_2, described in the following section), 200 μM each of dATP, dTTP, dCTP, dGTP, and 2 U Thermus auquaticus DNA polymerase (Perkin Elmer Cetus Instruments) were overlaid with 100 μl of liquid paraffin to prevent evaporation. Each of the 30 cycles of PCR consisted of incubations at 95°C for 1 min, at 50°C for 2 min, and at 72°C for 2 min except that the last extension period was for 7 min.

Screening of the cDNA Library

Approximately 80,000 bacterial colonies were screened with a DNA probe amplified from the lactating bovine mammary gland cDNA by PCR. First, a fragment of the bovine mammary gland cDNA was amplified by using degenerated oligonucleotide primers that were synthesized according to the MDGI protein sequence (8). Sequences of the primers are 5′ CAAGCTTAAQTTQGAQGAQTAQATQ 3′ designated as A_1 and 5′ GGGAATTQTGNCCPTTCCAQTTQTG 3′ designated as A_2 corresponding to amino acids at positions 16–21 (A_1) and 97–101 (A_2) of the described MDGI sequence. The oligonucleotides include additional restriction endonuclease sites for Hind III (A_1) and Eco RI (A_2) to facilitate ligation. The amplified fragment of 258 bp was isolated, cleaved with Eco RI and Hind III and cloned into pUC 19 for sequence analysis. The DNA probe was labeled by the random hexanucleotide primed synthesis method (21) resulting in specific activities of 1×10^9 cpm/μg of DNA. Colony hybridization was performed under low stringency conditions at 58°C in 500 mM $NaHPO_4$, pH 7.2, 1 mM EDTA, 1% SDS, 0.5% dry milk powder for 18 h. Duplicate nitrocellulose filters were washed three times for 20 min each in 0.1% SDS, 2× SSC at 50°C. (1× SSC: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.4).

Dot Blot Analysis

By use of the primers 5′ CAAGCTTGTNGAQGCNTTQGTN 3′ and 5′ GGGAATTCKATNACNGTPTCQCC 3′ (designated as B_1 and B_2, coding for residues 2–6 and 46–51, respectively) a MDGI fragment spanning amino acids 2–51 was amplified from both cDNA and genomic DNA by PCR. To this end 5 μl of each of the DNA amplified in vitro was spotted onto nitrocellulose filters. Oligonucleotide probes designated as SE, DK, LS, or TE that are either derived from the cDNA sequence (DK, TE) or from the protein sequence for MDGI (SE, LS) were used. The filters were soaked successively in 0.5 M NaOH, then in 1 M Tris, pH 8.0, and finally in 0.5 M Tris, pH 8.0, 1.5 M NaCl. Filters were baked for 2 h at 80°C under vacuum. For hybridization, the filters were prehybridized for 16 h at 52°C (for hybridization with TE/LS) and at 43°C (for hybridization with DK/SE) in 3.0 M tetramethylammonium chloride, 50 mM Tris, pH 8.0, 2 mM EDTA, 100 μg/ml of sonicated, denatured salmon sperm DNA, 0.1% SDS, and 5× Denhardt's solution. Hybridization conditions were chosen to detect single base mismatches (10, 40). Approximately 100 pg of ^{32}P-labeled oligonucleotide probe (sp act 10^9 cpm/μg) were added to nitrocellulose filters and hybridization was performed for 1 h at 43°C (DK/SE) or at 52°C (TE/LS), respectively. The filters were washed in 2× SSC, 0.1% SDS for 5 min at room temperature, followed by a 5-min wash at 45°C for (DK/SE) and at 54°C for (TE/LS) in 5× SSC, 0.1% SDS. Subsequently the filters were washed twice in hybridization buffer without Denhardt's solution and salmon sperm DNA and then incubated for 1 h in the same solution at 43°C for (DK, SE) and at 53°C for (TE, LS).

DNA Sequence Analysis

DNA from positive clones derived from the cDNA library or from the amplified and cloned PCR fragments was sequenced in both directions by the dideoxy chain-termination method (50), adapted for plasmid sequencing (12). Restriction fragments from each plasmid were subcloned for sequencing in M13 mp18 and mp19 Phage (43).

Northern Blot Analysis

Equal amounts of total cellular RNAs, isolated as described (14) were subjected to Northern blot analysis (36). The Northern blots were probed with the complete 681-bp cDNA fragment. Labeling and hybridization conditions were the same as for colony screening except that the washing temperature was 60°C. The relative intensity of hybridization signals in Northern blotting was estimated using a Shimadzu scanning densitometer.

In Situ Hybridization

The complete MDGI cDNA was subcloned in pGEM 3Z (Promega Biotech, Madison, WI). After linearizing the plasmid pGEM/MDGI with Hind III the insert was transcribed in vitro with T7 polymerase yielding a ^{35}S-labeled antisense RNA probe with a specific activity of 10^9 cpm/μg. Bovine mammary tissue at different stages of development was collected and stored frozen until sectioning. Gamma-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO) coated slides were used to lift 6-μm cryostat sections which were then fixed in freshly prepared 4% paraformaldehyde for 1 min and dehydrated in 70% ethanol. Hybridization was performed at standard conditions (41) with the following modifications. Prehybridization treatment consisted of rehydration of the sections in 2× SSC, acetylation in 0.25% acetic anhydrate, 0.1 M triethanolamine, pH 8.0, followed by immersing the slides for 30 min in 0.1 M Tris-HCl, pH 7.0, 0.1 M glycine. After rinsing the slides in 2× SSC and an incubation in 2× SSC, 50% formamide at 55°C for 5 min, the sections were finally hybridized in a solution composed of 2× SSC, 50% formamide, 10% dextran, 10 mM DTT, 1 μg/ml *E. coli* tRNA, 1 μg/ml sheared herring sperm DNA, 2 μg/ml BSA, and 1×10^6 cpm of ^{35}S-labeled RNA probe at 50°C for 3 h. The slides were then washed in 2× SSC, 50% formamide at 52°C for 30 min with three buffer changes, rinsed in 2× SSC, and then incubated in RNase solution (100 μg/ml RNase A, 1 μg/ml RNase T1) at 37°C for 30 min. After final washes in 2× SSC, 50% formamide at 52°C for 5 min, and 2× SSC at room temperature for 10 min, sections were dehydrated in 70, 80, and 90% ethanol. Hybridization of the tissue sections of the lactating mammary gland with the same amount of antisense ^{35}S-labeled RNA probes for the PDGF A-chain did not show any positive signal. Furthermore, if sections were pretreated with RNase solution before hybridization, no signal was detected. Sequential sections were also hybridized with an antisense β-actin RNA probe; positive signals were obtained, indicating the presence of intact RNA in the

examined tissue. Autoradiography was performed with a NBT-2 nuclear track emulsion (Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY) melted at 43°C and diluted 1:1 in water. The slides were exposed for 4–5 d in the dark at 4°C, and were then developed and counterstained with hematoxyline and eosine, dehydrated, mounted, and covered with coverslips.

Immunohistochemistry

Cryostat sections used for immunohistochemistry were stored at −20°C and fixed in cold acetone for 10 min just before use. Sections were preincubated with normal goat serum, then reacted with affinity-purified rabbit anti-MDGI-IgG (42) and stained by the ABC-technique using a biotinylated secondary antibody and preformed avidin and biotinylated horseradish peroxidase complex according to reference 29.

Normal rabbit serum was used as a negative control. Immunostained sections were photographed after hematoxyline staining with bright field optics.

Preparation of Antibodies

An affinity-purified anti-MDGI-IgG and an antibody directed against the peptide EFDETTADDR corresponding to amino acids 69–78 of MDGI (designated as anti-p69/78-IgG) has been described earlier (42). A second peptide antibody, anti-p121/131-IgG was obtained as follows. The peptide TAVCTRVYEKQ, corresponding to amino acids 121–131 of MDGI, was synthesized (42), reduced by β-mercaptoethanol and passed over Sephadex G10. BSA, activated by iodoacetamide in the presence of *N*-succinimidyl-bromo-acetate (4), purified by gel chromatography on Sephadex G25 M, was then conjugated with the reduced peptide under stirring for 2 h at room temperature. The conjugate was dialyzed against PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) at 4°C and then injected into rabbits. The resulting antiserum was purified by affinity chromatography on MDGI coupled to CNBr-activated Sepharose CL-4B (Pharmacia) as described (42). Western blot experiments were performed as described earlier (7).

Electron Microscopy

The procedure which has been described earlier (42) was slightly modified. Ultrathin sections from Lowicryl K4M embedded tissue were collected on formvarcarboncoated copper grids and were floated first on droplets of TBS supplemented with 16–48 μg/ml antibodies. For reducing the background the concentration of NaCl was increased to 500 mM and the washing buffers were supplemented with 0.1% BSA and 0.05% Tween 20. After washing five times for 2 min they were incubated for 30 min with protein A conjugated with gold particles (diameter 15 nm), (diluted with TBS/BSA to an absorbance of 0.04 at 525 nm).

The specificity of the labeling procedure was determined by replacing the different antibodies against MDGI by (*a*) TBS/BSA, (*b*) preimmune rabbit IgG, (*c*) "flow through"-IgG from a MDGI affinity column, and (*d*) anti-MDGI-IgG preadsorbed with MDGI. Immunoreactive staining was abolished under these conditions. Labeled ultrathin sections were conventionally poststained with uranyl acetate and lead citrate. Electron micrographs were taken with an EM 400T (Philips) at an acceleration voltage of 80 kV.

Results

Nucleotide Sequence of MDGI cDNA

Starting from the cDNA synthesized from RNA of lactating mammary gland a MDGI-specific probe was amplified by PCR. Amplification with degenerated oligonucleotide primers complementary to amino acids 16–21 (A_1) and 97–101 (A_2), which were deduced from the MDGI protein sequence, generated a 258-bp fragment. Sequence analysis showed that this fragment codes for the anticipated amino acids 16–101 of the reported MDGI amino acid sequence. However, at position 41 Leu was exchanged for Thr, a Ser was exchanged for Glu at position 44, and Gln was substituted by His at position 94. The bovine mammary gland cDNA library was then probed with this fragment under low stringency conditions. Nine cDNA clones were isolated and all of the nine isolated clones showed an insert length of ~680 bp. Nucleotide sequence analysis revealed identical sequences for all clones (Fig. 1). Translation of the compiled nucleotide sequence disclosed an initiation codon that begins at position 38 followed by an open reading frame that terminates with a TGA terminator codon at nucleotide 437. The 133 residues long polypeptide encoded by this region has a calculated molecular weight of 14,673. The ATG initiation site is integrated in the consensus sequence CCRCCATG proposed for eucaryotic translation initiation (35). The 3′ untranslated region spans 235 nucleotides including a consensus AATAAA polyadenylation signal 14 nucleotides upstream of the poly (dA)-tail. The open reading frame codes for most of the amino acids reported in the published sequence for MDGI (8) although differences were found at six positions. In positions

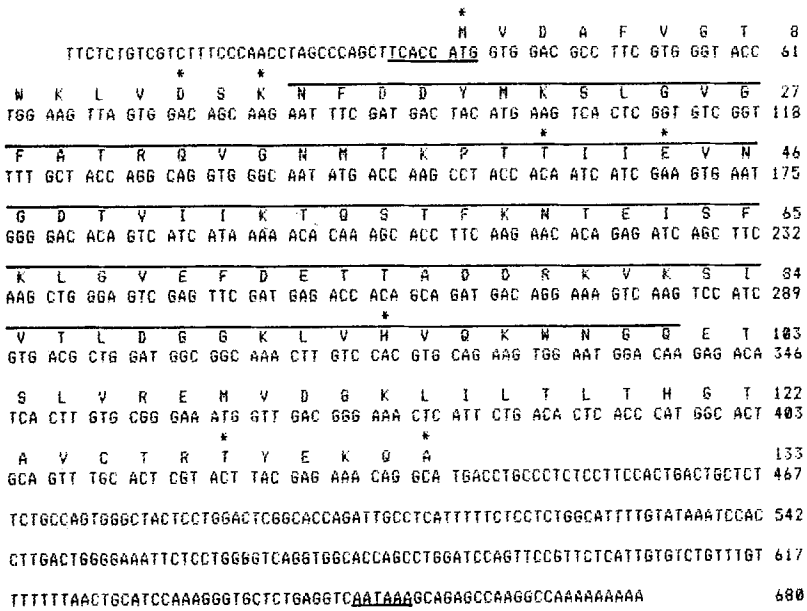


Figure 1. Nucleotide and deduced amino acid sequence of MDGI cDNA. Residues that are superlined correspond to the amplified fragment used as screening probe. The nucleotide sequence underlined at the beginning of the open reading frame represents the eucaryotic translation initiation consensus sequence. The polyadenylation signal in the 3′ nontranslated region is also underlined. Amino acids that are not identical with those of the reported MDGI sequence are labeled by asterisks. These sequence data are available from EMBL/GenBank DDBJ under accession number X51933.

41 and 44 the cDNA codes for Thr and Glu, respectively, instead of Leu and Ser, which were found in amino acid sequence analysis. The other differences at position 13 (Asp for Ser), 15 (Lys for Glu), 94 (His for Gln), and 128 (Thr for Val) represent ambiguous determinations in the reported protein sequence, where the amino acids predicted from the cDNA sequence were also found, but at lower yield (8). The Met residue at position 1 and the Ala at position 133 of the cDNA deduced sequence were not found by protein sequencing and are possibly removed by posttranslational protein processing.

Dot Blot Analysis for MDGI Sequence Variants

To investigate the possible appearance of nonabundant cDNA species coding for MDGI the approach of selective hybridization with a collection of synthetic oligonucleotides was chosen (Fig. 2). To this end PCR was performed with primers constructed to amplify fragments corresponding to the cDNA-deduced MDGI structure or to fragments containing putative exchanges in positions 13, 15, 41, or 44. DNA fragments overlapping amino acids 2–51 of MDGI were amplified from cDNA of two separate bovine mammary glands (Fig. 2, *a* and *b*) and from bovine genomic DNA of three different animals (Fig. 2, *c–e*). 5 μl aliquots of the amplified DNA samples were spotted on nitrocellulose and hybridized with oligonucleotide probes; probes DK and TE coded for amino acids VDSKN and TTIIEV, respectively, and were derived from the cDNA sequence, and probes SE and LS corresponded to amino acids VSSEN and TLIISV, respectively, which were determined by amino acid sequence analysis. Each of the peptides contain two amino acid exchanges generating at least two base pair exchanges between DK/SE and four between TE/LS. Hybridization was performed under conditions where only completely matched hybrids remain stable. Under these conditions, only the oligomers DK and TE that are complementary to the described cDNA sequence, hybridized with the amplified DNA. No hybridization was obtained with the oligomers SE and LS derived from the protein sequence.

Northern Blot Analysis

To determine the relative level of MDGI mRNA in different bovine tissues and in bovine mammary glands of different functional state a Northern blot analysis using the complete MDGI cDNA was performed (Fig. 3). A transcript of 0.7 kb was detected in the mammary gland of midpregnant (Fig. 3 *b*), and lactating animals (Fig. 3 *c*), in heart muscle tissue (Fig. 3 *e*), and in skeletal muscle (Fig. 3 *g*). No MDGI mRNA was found in liver (Fig. 3 *f*) and pancreas (Fig. 3 *h*).

The MDGI mRNA level in mammary gland depends on the developmental stage of the tissue. In virgin mammary tissue MDGI mRNA is not present (Fig. 3 *a*), its level increases during pregnancy (Fig. 3 *b*) reaching a maximum at lactation (Fig. 3 *c*). From densiometry the increase of MDGI mRNA was fivefold at lactation in comparison to pregnancy. Also, there is a geographical gradient of MDGI mRNA starting from a high concentration in proximal parts of lactating mammary tissue (Fig. 3 *c*) and declining to zero in distal parts (Fig. 3 *d*).

Localization of MDGI Gene Transcription in the Mammary Gland by In Situ Hybridization

Based on the findings that transcription of MDGI is related to the proliferative state of mammary gland, cryosections were analyzed for MDGI mRNA using in situ hybridization technique. The results obtained with a ^{35}S-labeled antisense RNA probe are summarized in Figs. 4 and 5.

To follow MDGI transcription during development tissue sections were taken from embryonic, virgin, pregnant, and from different areas of lactating mammary gland. Analysis of a 5-mo-old female bovine embryo revealed clearly detectable transcripts in the mammary epithelial rudiment (Fig. 4, *A* and *B*). MDGI mRNA is evenly distributed with similar amount over the distal and proximal regions. Mesenchymal

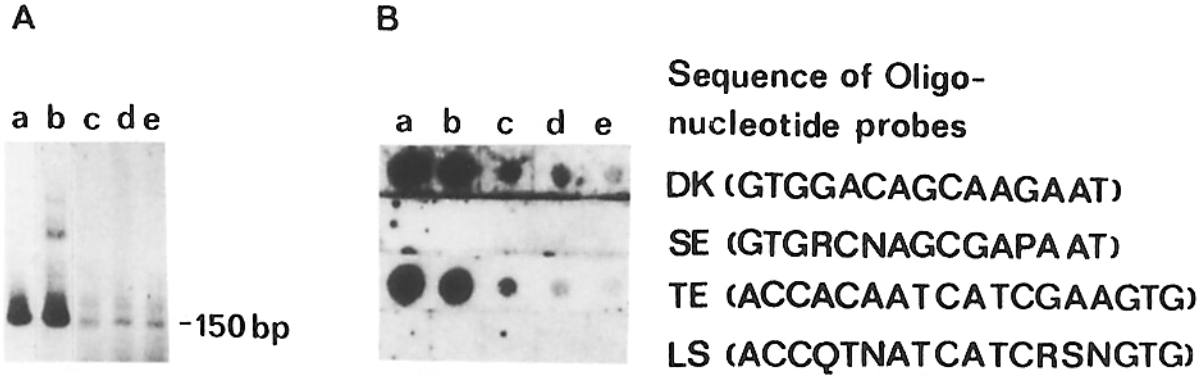


Figure 2. Dot blot analysis of amplified MDGI sequences. The amplification of MDGI fragments by PCR is shown in *A*. cDNA from two distinct animals (*a* and *b*) and genomic DNA from three bovine breeds (*c–e*) were used to amplify a fragment of 150 bp. Sequence analysis proved this fragment to code for amino acids 2–51 of reported MDGI amino acid sequence thus confirming the MDGI cDNA. Dot blot analysis of the amplified DNA samples is shown in *B*. Analysis was performed by use of oligonucleotide probes either hybridizing specifically with the MDGI cDNA sequence (*DK*, *TE*) or with the MDGI protein-derived sequence (*SE*, *LS*). Oligonucleotides DK and SE are spanning the region between amino acids 12–16 and oligonucleotides TE and LS the region between amino acids 40 and 45 of MDGI. Each region contains two amino acid differences. About 50 ng of the amplified fragment were spotted on nitrocellulose and hybridized to the oligonucleotide probes for detection of single base substitutions. An autoradiogram of filters exposed for 4 h is shown. No signal could be detected with the probes SE and LS, even after 1 wk of autoradiography.

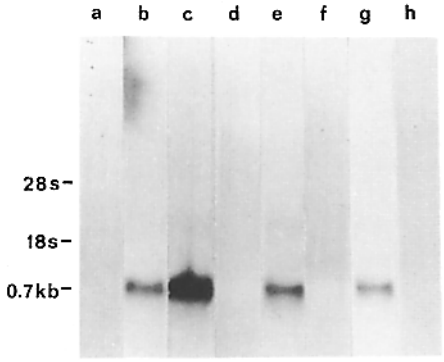


Figure 3. Northern blot analysis of bovine tissue RNAs. Hybridization was performed with the complete MDGI cDNA. Blots were prepared with total cellular RNA (20 μg) from virgin mammary tissue (*a*), pregnant mammary tissue (*b*), central parts of lactating mammary tissue (*c*), peripheral parts of lactating mammary tissue (*d*), heart (*e*), liver (*f*), red skeletal muscle (*g*), and pancreas (*h*). Exposure time, 48 h.

tissue was not labeled by the antisense RNA probe (not demonstrated). In the immature, resting mammary gland of the virgin animal, MDGI mRNA was not detected (Fig. 4 *C*). The bovine mammary gland in the fifth month of pregnancy is undergoing deep morphological and functional changes (Fig. 4, *D* and *E*). In Fig. 4 *D* a branching duct is shown which terminates with developing lobuloalveoli. During this stage of development ductal and alveolar epithelial cells in combination with myoepithelial cells are forming the lobuloalveolar gland. In the ductal epithelial cells, MDGI transcription was rather low while epithelial cells of developing alveoli contained MDGI transcripts. Analysis of a developing lobulus at higher magnification clearly shows that MDGI transcription is enhanced in the alveolar cells when compared to ductal epithelial cells (Fig. 4 *F*). Closer analysis of the lobuloalveolar gland of the pregnant animal revealed an increased MDGI mRNA level in the alveolar epithelial cells bordering on the connective tissue (not shown). Other cell types such as myoepithelial cells and connective tissue were not transcribing MDGI.

The terminally differentiated lactating mammary gland is characterized by ducts branching into large secretory active lobuloalveolar structures (Fig. 5, *A–C*). In contrast to the pregnant stage both the alveolar (Fig. 5, *A* and *B*) and ductal cells (Fig. 5 *C*) of terminally differentiated mammary gland transcribe the MDGI gene. Along the duct, a striking increase in the level of MDGI transcripts was found with highest levels in the terminal part (Fig. 5 *C*). In general, MDGI mRNA was clearly expressed at higher levels in the ductal epithelial cells compared to the alveolar mammary epithelial cells. Other cell types such as fibroblasts and myoepithelial cells were not found to transcribe the MDGI gene. MDGI transcription follows a geographical gradient; in the less differentiated distal parts of lactating mammary gland (Fig. 5 *D*) including the nipple of the mammary gland (Fig. 5 *E*) MDGI mRNA was clearly reduced. During all stages of differentiation MDGI protein expression parallels the MDGI mRNA level. For example, Fig. 5 *F* shows an immunohistochemical analysis of MDGI expression by using an affinity-purified rabbit anti-MDGI antibody. The picture also shows secretion of MDGI.

Immunohistochemical Analysis of MDGI Distribution in the Mammary Gland

Specificity of Antibodies. A high speed supernatant of a homogenate of lactating bovine gland was subjected to SDS-PAGE and immunoblotting (Fig. 6). A single band corresponding to an antigen of 14.5 kD was identified with the purified polyclonal anti-MDGI-IgG, as well as with anti-p69/78-IgG, an antiserum made against a peptide comprising amino acids 69–78, and with anti-p121/131-IgG, made against a peptide comprising amino acids 121–131. Preincubation of the antibodies with an excess of MDGI led to quenching of the staining of the 14.5-kD band, indicating that the reactions were specific.

Intracellular Distribution of MDGI in Mammary Gland of Different Functional State. Lowicryl-embedded ultrathin sections taken from virgin, pregnant, and lactating bovine mammary gland were analyzed by means of the immunogold technique using affinity-purified anti-MDGI-IgG. An increasing density of immunolabeling was detected over resting, proliferating, and differentiated mammary epithelial cells (Fig. 7), which is in accordance with the MDGI mRNA levels determined before in the same tissues. In the virgin tissue MDGI was not expressed (Fig. 7 *A*), a moderate intensity of immunolabeling was found during pregnancy (Fig. 7 *B*), reaching a maximal level during functional differentiation of the mammary gland (Fig. 7 *C*).

Immunolabeling of mammary epithelial cells was associated with basal invaginations, the cytosol, and the transcriptionally active euchromatic regions of nuclei. Other cell compartments such as mitochondria and rough endoplasmic reticulum were not labeled. A very similar though less intense staining pattern was observed with the two antisera against peptides of MDGI (Fig. 7, *D* and *E*). Some preferential nuclear labeling was observed with the antibody directed against the COOH-terminal amino acids 121–131 of the MDGI sequence (Fig. 7 *E*).

Discussion

In this communication we report on the cDNA cloning for MDGI and the developmental expression of MDGI mRNA in bovine mammary gland. On the basis of amino acid sequence analysis we have shown earlier that MDGI belongs to a family of structurally related proteins, consisting of fatty acid binding proteins (1, 6, 15, 44), cellular retinoid binding proteins (13, 37), myelin P-2, and an adipose differentiation dependent protein, called P422 (5, 34, 38). MDGI is also immunologically related to a fibroblast growth inhibitor (9). The amino acid sequence deduced from the cDNA sequence and the protein-derived amino acid sequence differ in six positions, not taking into account a NH_2-terminal methionine and a COOH-terminal alanine which are thought to be removed by posttranslational modification.

In each of positions 12, 14, 93, and 127 protein sequencing yielded two amino acid residues (8). At these positions the cDNA-deduced residues correspond to the amino acids present at lower yield. Two additional differences were detected at positions 40 and 43, where the MDGI cDNA predicts Thr and Glu instead of Leu and Ser, respectively. The differences might have arisen from genetic polymorphism of the animals, although mutations in the intraspecies variant between

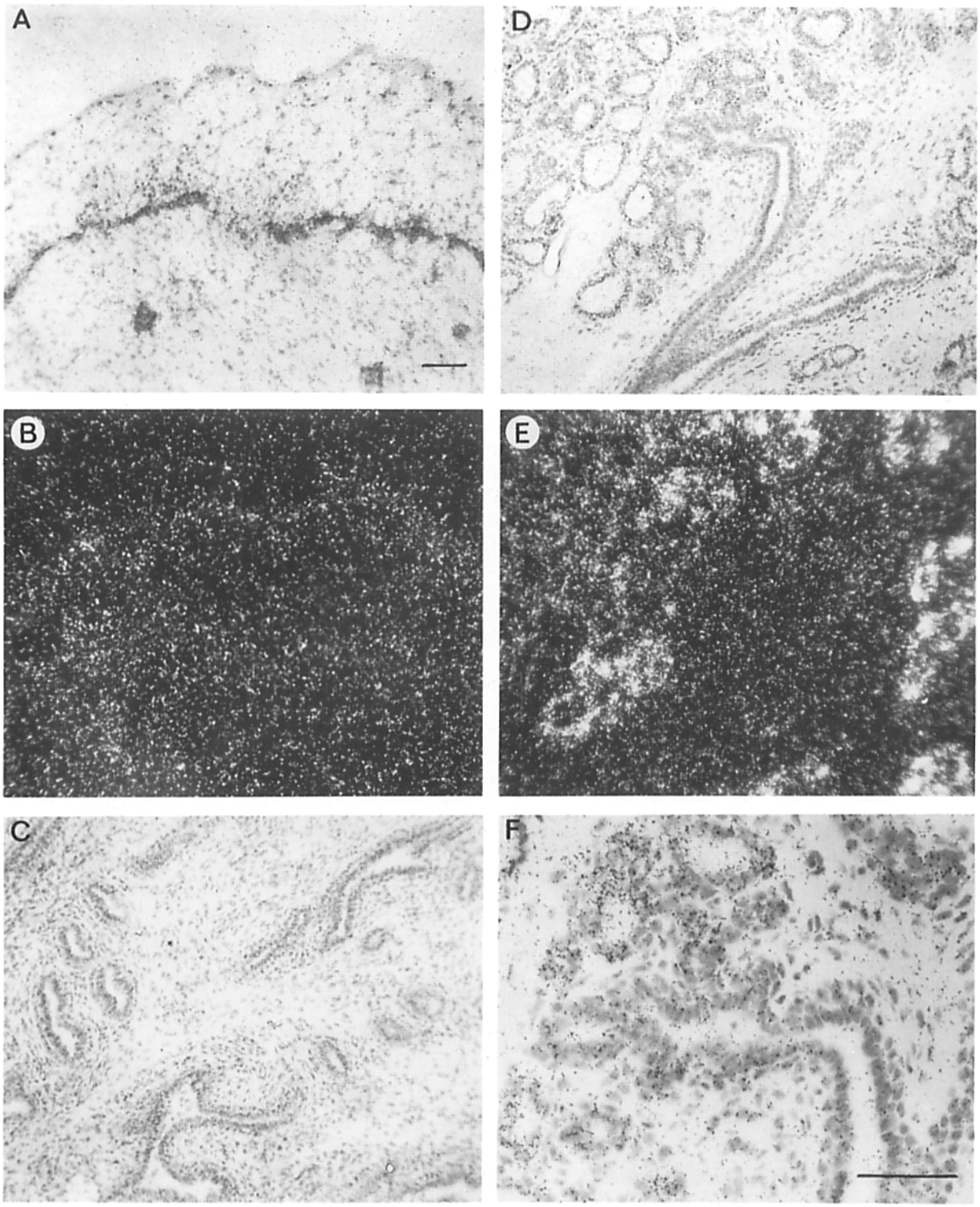


Figure 4. Distribution of MDGI RNA in embryonic, virgin, and pregnant mammary tissue. MDGI transcripts were detected by in situ hybridization, using cryostat sections hybridized with ^{35}S-labeled antisense RNA probe. Shown are bright-field and dark-field pictures of sections counterstained with hematoxyline. (*A* and *B*) Sections of mid stage mammary epithelial rudiment; MDGI transcripts are evenly distributed in epithelial cells of the embryonic mammary rudiment. (*C*) Virgin mammary gland; no MDGI transcripts are detectable. (*D* and *E*) Longitudinal sections of midpregnant mammary gland. This picture shows the terminal part of a duct where lobuloalveolar structures are developing. (*F*) Higher magnification of a part of section shown in *D*. Epithelial cells of the lobuloalveoli are expressing MDGI mRNA at this early stage of development. In contrast, epithelial cells of the duct are not labeled. Bars, 100 μm.

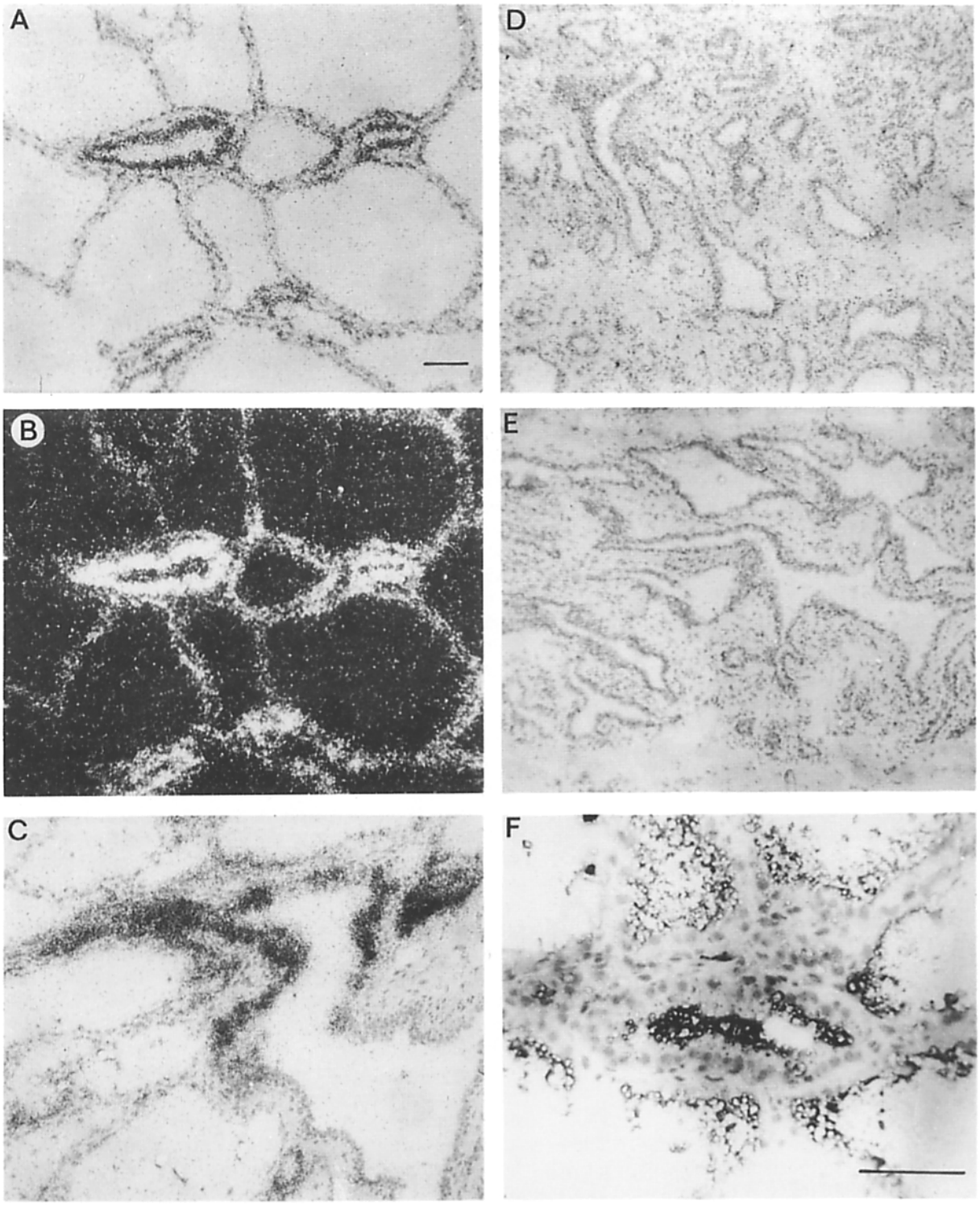


Figure 5. Distribution of MDGI mRNA in terminally differentiated bovine mammary gland. (*A*) Cross section of proximal part of lactating mammary tissue; (*B*) dark-field picture of *A*; (*C*) longitudinal section of tissue in the same developmental stage as in *A*; (*D*) distal part of the same lactating mammary tissue; and (*E*) section of the nipple of the same tissue. (*F*) Section of tissue shown in *A* stained by the ABC method with affinity-purified polyclonal anti-MDGI IgG. Bars, 100 μm.

the two residues would have required the replacement of four bases in the codons. Moreover, including RNA preparations from different animals, both screening under low stringency conditions of the cDNA bank, followed by sequence analysis of nine clones, and dot blot analyses of fragments amplified from either cDNA or genomic DNA did not reveal any polymorphism. Nevertheless, the techniques used in the present paper do not rule out the possibility that slightly different forms of MDGI exist in the mammary gland. In this regard, several reports have discussed multiple forms of fatty acid

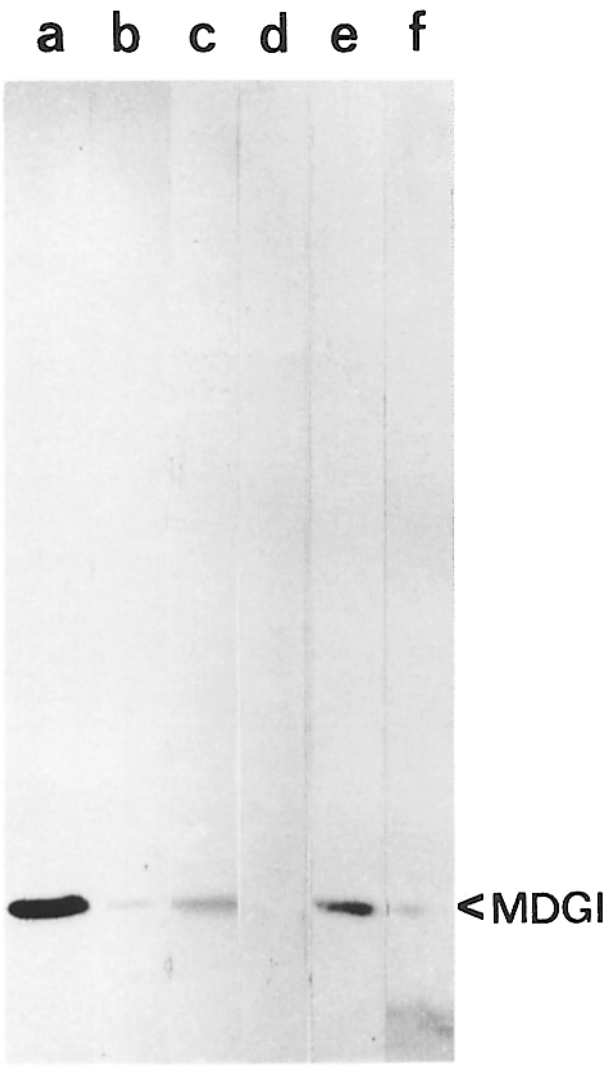


Figure 6. Specificity of anti-MDGI-antibodies. A high speed supernatant of a homogenate from lactating bovine mammary gland was subjected to SDS-PAGE. Immunoreactive bands were detected by immunoblotting and stained using anti-MDGI-IgG (lanes *a* and *b*), anti-p69/78-IgG (lanes *c* and *d*), and anti-p121/131-IgG (lanes *e* and *f*). Controls were immunostained with anti-MDGI-IgG preadsorbed with MDGI (lane *b*), with anti-p69/78-IgG preadsorbed with MDGI (lane *d*), and with anti-p121/131-IgG preadsorbed with MDGI (lane *f*).

binding proteins (FABPs) in heart (15, 28, 34, 44, 47), intestine (1, 3), liver (26, 54), and mammary gland (8, 33, 61).

For the bovine heart FABP (6) two isoforms were found which differ by one exchange (Asn for Asp at position 98) from each other. We also have detected at least two MDGI-forms in bovine mammary gland differing in their isoelectric points (Böhmer, F.-D., unpublished results). Whether these forms are coded by different genes is not known. Chromosome mapping study revealed that the murine genome contains at least three loci located on different chromosomes which hybridize with a cloned heart FABP cDNA (60). Whether these loci represent pseudogenes or transcribing sequences of homologous proteins could not be determined.

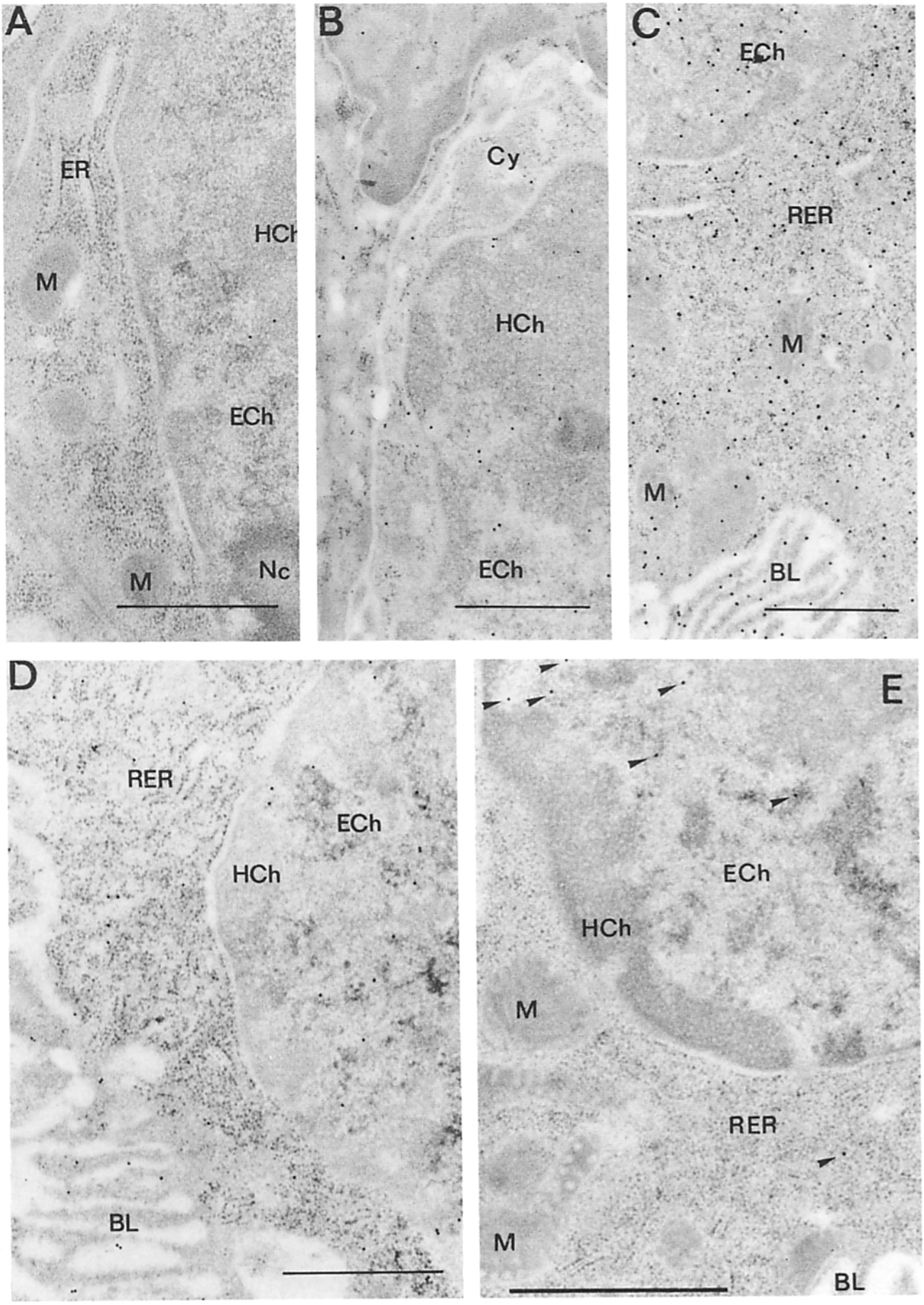
The cDNA-deduced MDGI sequence is identical with that reported for the bovine heart FABP (6). Thus, the MDGI mRNA is expressed in heart tissue. The exact function of FABPs is not yet identified. It has been suggested that in the heart, FABP regulates the supply of fatty acids to the mitochondria for beta-oxidation (3, 6). The mammary gland, however, is a highly lipogenic tissue and fatty acids are not likely to be a major fuel for its metabolism. Therefore, MDGI could fulfill different functions in heart and in mammary gland. The functional meaning of heart muscle related FABPs in brain (3, 28, 51) and kidney (3, 15) is for similar reasons difficult to understand. It is possible that different forms of FABPs and MDGI fulfill several unrelated functions, including growth inhibition. In this regard, it has been established recently that some FABPs are expressed in a differentiation dependent manner (16, 46, 52, 54). For cellular retinoic acid binding protein (13, 19, 37) and P422 (5) this was described earlier. It seems clear that a differentiation associated function is a common property of these structurally related proteins. For example, expression of the rat intestinal FABPs shows a distinct gradient from the proximal to the distal intestine and from crypts to villus tips (23, 54). The authors conclude that the FABP gene products are sensitive indicators of differences in fetal intestinal epithelial cell differentiation (23).

Similar to the expression of intestinal FABP, MDGI transcription follows a geographical gradient with higher mRNA levels towards the proximal glandular tissue. There was a distinct difference in expression in midpregnant and terminally differentiated tissue. During midpregnancy, when the mammary epithelium undergoes deep functional changes and lobuloalveolar structures emerge, MDGI transcription was mainly restricted to alveolar epithelial cells bordering connective tissue. These areas are characterized by a higher proliferative rate before entering the differentiation phase (18). In contrast, during functional differentiation in the lactating gland, MDGI transcripts were found predominantly in epithelial cells lining the terminal part of ducts. We have shown earlier that during differentiation MDGI mRNA increases about fivefold comprising 0.5–0.8% of total mRNA in lactation (42).

In summary, the data suggest that MDGI transcription is related to some functional role of MDGI in the onset of early differentiation which is coupled to inhibition of cell proliferation. In this respect, we found that injection of lactogenic hormones known to induce differentiation increase the MDGI mRNA level in hypophysectomized rats (Kurtz, A., unpublished results). The appearance of MDGI transcripts in embryonic mammary epithelium also indicates a hormonal regulation of MDGI expression.

The apparent close parallel between expression of MDGI and of its mRNA during normal development of the bovine mammary gland suggest that transcriptional control mecha-

Figure 7. Immunolabeling of epithelial cells of mammary glands in different functional states. Lowicryl K4M embedded sections of virgin (*A*), pregnant (*B*), and lactating (*C*) animals were incubated with anti-MDGI-IgG. Sections of terminally differentiated epithelial cells of lactating glands were also analyzed with anti-p69/78-IgG (*D*) and anti-p121/131-IgG (*E*). Euchromatin (*ECh*), heterochromatin (*HCh*), mitochondria (*M*), endoplasmic reticulum (*ER*, *RER*), basal lamina (*BL*), cytoplasma (*Cy*). Bars, 1 μm.


A
ER
HCh
M
ECh
M
Nc
B
Cy
HCh
ECh
C
ECh
RER
M
M
BL
D
RER
ECh
HCh
BL
E
ECh
HCh
M
RER
M
BL

nisms are a major regulator. Taking into account our finding of a chromatin-associated MDGI-form (42) it is intriguing to speculate that under conditions of an increased MDGI synthesis in mammary epithelial cells entering differentiation, MDGI is directly involved in gene expression. The availability of the MDGI cDNA will now make it possible to define more exactly the role of MDGI during the development of mammary gland by performing expression studies coupled with site-directed mutagenesis.

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