Isolation and Culture of Bovine Mammary Epithelial Cells and Establishment of Gene Transfection Conditions in the Cells

Jung-Youb AHN, Naohito AOKI, Takahiro ADACHI, Yuri MIZUNO, Ryo NAKAMURA, and Tsukasa MATSUDA

Department of Applied Biological Sciences, School of Agricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

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Bovine mammary epithelial cells (BMEC) were isolated as acinous fragments from a mammary gland of a lactating cow. They grew well on plastic substratum, showed the characteristic cobbledstone morphology of epithelial cells, and secreted α1-*, β-, and κ-caseins even when grown on plastic substratum. A plasmid containing the bacterial chloramphenicol acetyl transferase (CAT) gene was transfected to the isolated BMEC by calcium phosphate precipitation and electroporation methods. The transfection efficiency of BMEC by the calcium phosphate method was greatly improved by post-transfection osmotic shock with glycerol or polyethylene glycol. An about 700 bp DNA fragment containing 5'-flanking sequence of bovine α1-casein gene showed promoter activity in the transfected BMEC. The primary culture of BMEC might be useful for studies on regulation of bovine milk-protein gene expression.

Mammary epithelial cells synthesize and secrete a large amount of proteins, and such milk protein synthesis as well as cell growth and differentiation are regulated by a variety of factors including peptide and steroid hormones, cell-substratum, and cell-cell interactions. The molecular mechanism of developmentally and hormonally regulated expression of milk protein genes has been investigated mostly on primary culture and some cell lines of murine mammary epithelial cells. The gene expression of the major milk protein, casein, has been reported to be regulated by the synergistic actions of insulin, glucocorticoid, and prolactin. Regulatory sequence elements of murine casein genes, especially the β-casein gene, have also been investigated using some mouse cell lines and transgenic mice. On the other hand, milk protein gene expression of cattle, a large domestic ruminant, has not been well investigated. Transcriptional regulation of the bovine β-casein gene was investigated using a mouse mammary epithelial cell line, C1D9. However, biological functions of bovine mammary glands would not necessarily be identical to that of a non-ruminant experimental animal, such as the mouse. For more precise investigation of the regulation mechanisms of bovine mammary-specific gene expression, experimental systems with homologous combination of genes and cells from bovine are required. Primary culture of ruminant mammary epithelial cells that synthesizes and secretes milk constituents have been prepared in only a few laboratories.

We have therefore prepared primary cultures of mammary epithelial cells from a lactating cow. In this paper, we describe transfection of an SV40 early gene promoter-chloramphenicol acetyl transferase (CAT) chimeric gene in the cultured bovine mammary cells by calcium phosphate precipitation and electroporation methods under various transfection conditions. The expression of bovine α1-casein-CAT chimeric genes in the bovine mammary cells transfected under the optimum condition is also reported.

Materials and Methods

Cell preparation and culture. Bovine mammary epithelial cells (BMEC) were isolated from the mammary gland of a lactating Holstein cow obtained from a local slaughterhouse, by the method of Enami et al., with slight modification. Briefly, the mammary tissue was minced with a surgical knife, and the tissue pieces were dissociated in Hank's balanced salt solution (BSS) containing 0.05% trypsin (Sigma) with gentle shaking at 37°C for 14h. After filtration with a stainless steel mesh to remove undissociated tissues and debris, the cells were collected by low speed centrifugation at 80 × g for 3 min. The cells were further dissociated in Hank's BSS containing 0.05% pronase (Sigma) with gentle shaking at 37°C for 30 min. The cells were collected by centrifugation at 80 × g for 1 min, and washed three times with Hank's BSS.

The BMEC preparation was suspended in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, penicillin at 10 unit/ml, streptomycin at 5 µg/ml, and fungizone (GIBCO) at 50 mg/ml (basal medium), seeded at a density of 5 × 10⁶ cells, and cultured on 9 cm plastic dishes at 37°C under 5% CO₂ and 95% air. For hormonal induction of protein synthesis, the cells were cultured in the presence of bovine insulin (5 µg/ml; Sigma), hydrocortisone (1 µg/ml; Sigma), and ovine prolactin (4 µg/ml; Sigma) as indicated.

Radioisotopic labeling and protein analysis. BMEC were seeded into 24-well culture plates (Falcon) at a density of 5 × 10⁵ cells/well, and cultured in the basal medium for 2 days. The culture medium was replaced with fresh basal medium with or without the lactogenic hormones, and the cells were cultured for 2 days. The BMEC were then washed once with methionine-free MEM (Sigma) and incubated in 200 µl of the methionine-free MEM containing 18.5 Mbbq (0.5 mCi) [³⁵S] methionine (Du Pont) in the presence and absence of the lactogenic hormones at 37°C for 4h under 5% CO₂ and 95% air. The radio-labeled proteins secreted into the culture medium were collected by trichloroacetic acid precipitation. The labeled proteins were separated by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis as described by Laemmli, and the gel sheets were treated with EN³HANCE (Du Pont), dried and exposed to X-ray film at ~80°C.

Immunoblotting analysis. The culture supernatants of BMEC were

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* Corresponding author.

Abbreviations: BMEC, bovine mammary epithelial cell; CAT, chloramphenicol acetyl transferase; BSS, balanced salt solution; DMEM, Dulbecco's modified Eagle medium; PCR, polymerase chain reaction; CN, casein.
dialyzed against distilled water, and precipitated proteins including caseins were collected by centrifugation and analyzed by immunoblotting as described previously.\textsuperscript{14} The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore).\textsuperscript{15} The membrane was blocked with 3% bovine serum albumin (Fraction V, Sigma) in phosphate buffered saline (PBS), and then incubated with rabbit antisera raised against α\textsubscript{1}-, β-, and κ-caseins and with peroxidase-coupled anti-rabbit IgG (Cappel), successively. The antisera specific for each casein component were kindly provided by Dr. H. Okumi (Shinshu University). The protein bands reactive to the specific antibody were activity-stained for peroxidase using 4-chloro-1-naphthol (Bio-Rad).

Construction of CAT plasmid. A plasmid containing CAT with the SV40 early gene promoter, pSV2CAT, was kindly provided by Dr. Y. Kitagawa, Nagoya University BioScience Center, and used as a control for the CAT assay.\textsuperscript{15} To construct the plasmids containing a casein-CAT fusion gene, the 5′-flanking regions of bovine casein and β-casein (x\textsubscript{2}CN and βCN) genes were amplified from bovine genomic DNA by polymerase chain reaction (PCR).\textsuperscript{16} For the PCR reaction, synthesized oligonucleotide primers were used as follows:

i) x\textsubscript{2}CN, 5′-CTGCAGTCCATGCGGGTC-3′, 3′-TAGTTCAGAC- TAGTAGGTG-5′ (position: −681 to −663/+1 to +18)\textsuperscript{17}

ii) βCN, 5′-AATACCTAGCTAAGAACATT-3′, 3′-TAATGCGAG- GAGAAGTGAA-5′ (position: −910 to −910/+1 to +20)\textsuperscript{18}

Each product of PCR was inserted into the HindIII site of pUC19, and an about 1.6-kb HindIII/BamHI fragment of CAT from pSV2CAT was fused downstream to the gene promoters. The 1.6-kb CAT fragment was also inserted into pUC19, and used as a control plasmid for transfection. Plasmid DNAs were prepared by ceasire chloride-ethidium bromide equilibrium gradient centrifugation.\textsuperscript{19}

Gene transfection. BMEC were seeded at a density of $1 - 2 \times 10^6$ cells/90 mm culture dish, and grown overnight in the basal medium. The cells were transfected with 20 μg of plasmid DNA in 1 ml solution by the calcium phosphate-DNA precipitation technique.\textsuperscript{20} After incubation for 12 h, the transfected cells were washed with PBS to remove the remaining calcium phosphate-DNA precipitate. At this stage, culture medium of each dish was replaced with 1.5 ml of HEPEs buffer (pH 7.5) containing 10% DMSO, 15% glycerol, or 11% or 44% PEG1500 (Wako), and the cells were kept in each solution for 1 to 6 min for osmotic shock treatment.\textsuperscript{21 - 23} The medium was then replaced with fresh basal medium, and the cells were incubated for 2 days at 37°C under 5% CO\textsubscript{2} and 95% air.

Gene transfection by electroporation was done using a Gene Pulser (Bio-Rad) with a capacitance extender, using Gene Pulser disposable cuvettes with a 0.4 cm electrode gap containing a final volume of 400 or 800 μl of cell-DNA mixture.

CAT assay. The CAT assay was done by the reported method\textsuperscript{15} with a slight modification. After 2 days of incubation following the gene transfection by either calcium phosphate or electroporation method, the cells were harvested by trypsinization (2 mm EDTA, 0.025% trypsin). The harvested pellet was re-suspended with 100 μl of 10 mm Tris-HCl buffer (pH 7.8) in Eppendorf tubes. Then the cells were ultrasonicated for 20 sec, 3 times. The samples were heated for 10 min at 60°C and centrifuged at 10,000 × g for 5 min at room temperature. To assay CAT activity, 80 μl of cell extract was mixed with 30 μl of 1 M Tris-HCl (pH 7.8), 5 μl of 5 mm acetyl CoA (freshly prepared, Wako) and 5 μl [0.463 MBq (12.5 μCi)/ml] of [14C]-chloramphenicol (ICN). The reaction was done for 3 h at 37°C; acetyl CoA was added every hour. Chloramphenicol was extracted with 0.5 ml ethyl acetate. After the solvent was dried out, the pellet was redisolved in 20 μl of ethyl acetate and spotted on a silica gel thin-layer plate (Merck Kieselgel 60) to separate the native chloramphenicol from its acetylated derivatives. Migration was done in chloroform-methanol (19:1) for 20 min. CAT activity was analyzed by an Image analyzer (Fujix BAS2000) and calculated as percent conversion of the substrate to 1- and 3-acetylated forms per total substrate.

**Fig. 1.** Phase Contrast Microscopic Observation of Bovine Mammary Epithelial Cells Cultured on Plastic Dishes.

The cells were prepared as described under Materials and Methods and cultured in DMEM containing 10% FCS, penicillin at 10 unit/ml, streptomycin at 5 mg/ml, and fungizone at 30 μg/ml (basal medium). A, acinuous fragments (day 0 after seeding, × 100 magnified); B and C, adhering, spreading, and growing cells (day 4 after seeding, ×100 and ×200 magnified, respectively); D and F, the characteristic cobblestone morphology (day 10 after seeding, ×100 and ×200 magnified, respectively); E, the contaminating fibroblast-like cells separated from the BMEC culture (× 200 magnified).
Results

I. BMEC preparation and their characterization

BMECs were efficiently prepared as acinous fragments from fresh mammary glands by a collagenase/Pronase dissociation method and low speed centrifugation. The phase contrast microscopic observation of the cells is shown in Fig. 1. Right after the preparation the cells did not appear singly but as clumps of 50–100 cells, so-called “organoids” (Fig. 1-A). About 4 to 5 days were required for the cells to start adhering, spreading, and growing on the plastic substratum (Figs. 1-B and 1-C). The cells grew well in the basal medium, and showed the characteristic cobblestone morphology of epithelial cells (Figs. 1-C, 1-D, and 1-F). The initial preparation was contaminated with fibroblast-like cells (Fig. 1-E), though the contaminating cells were removed effectively by low speed centrifugation during several passages. Since the mammary epithelial cells could easily be distinguished from the fibroblast-like cells under phase contrast microscopic observation (Figs. 1-E and 1-F), the purity of epithelial cells were estimated by counting the cells in 5 independent circles with 3 mm diameter under microscopic observation. The proportion of contaminating fibroblast cells against mammary epithelial cells was less than 5 percent in BMEC after four passages.

Proteins synthesized and secreted by cultured BMEC were also analyzed by radio-tracer experiments. To find whether the cultured BMEC still retain their ability to respond to lactogenic hormones added to the medium, cells were cultured on a plastic substratum in the basal medium with or without three lactogenic hormones, prolactin, hydrocortisone, and insulin, and proteins secreted into the culture medium were analyzed. Figure 2 shows the autoradiogram of proteins secreted by the cultured BMEC. The synthesis and secretion of several proteins were induced by the presence of all three hormones. Especially, the synthesis of about 32-kDa protein was strongly induced. This 32-kDa band was assumed to be α₁-casein based on the electrophoretic mobility. Thus, the cultured BMEC retained their sensitivity to hormones at least during several passages, though such sensitivity was largely dependent on batches of BMEC isolated from mammary glands of different lactating cows.

The secretion of milk proteins by the BMEC cultured in the basal medium with lactogenic hormones was examined by immunoblotting using specific antibodies. As shown in Fig. 3, not only α₁-casein but also β- and κ-caseins were clearly detected in the BMEC culture supernatant. The major milk whey proteins such as β-lactoglobulin and α-lactalbumin, however, were not detected. The weak bands with lower mobility observed in the lanes of β- and κ-caseins appeared to be unknown proteins in FCS, since the bands were also detected by these antisera in the control sample prepared from the basal medium without BMEC (data not shown).

II. Gene transfection by calcium-phosphate precipitation and electroporation methods

To investigate efficiency of transient transfection and expression of foreign genes in BMEC, a plasmid pSV2CAT was used as a model gene. The calcium phosphate precipitation method was first used for gene transfection. As shown in Fig. 4, the level of CAT expression in transfected BMEC greatly varied according to the transfection conditions. Only a slight or almost no CAT activity was detected in the cells transfected without osmotic shock treatment. However, CAT activity was increased by

![Fig. 2. Protein Synthesis and Secretion by BMEC Cultured in the Presence of Lactogenic Hormones.](image)

BMEC were cultured in the basal medium with the lactogenic hormones (hydrocortisone, prolactin, and insulin (lane 2) or without the hormones (lane 3), and then treated as described under Materials and Methods. Trichloroacetic acid insoluble proteins of the culture supernatant were separated by SDS-PAGE (12.5% acrylamide) and then autoradiographed with enhancement. Bovine milk proteins and molecular mass standards are shown in the lanes 1 and 4, respectively, for comparison. A. α₁-casein; B. β-casein; K. κ-casein; LG. β-lactoglobulin; LA. α-lactalbumin.

![Fig. 3. Immunological Detection of Milk Casein Components Synthesized and Secreted by Cultured BMEC.](image)

Confluent culture of BMEC on plastic dish (90 mm in diameter) were treated with lactogenic hormones (prolactin, hydrocortisone, and insulin) for 2 days. The culture medium was collected and dialyzed against distilled water at 4°C followed by centrifugation. Recovered proteins were separated by SDS-PAGE following with immunoblotting using the sera specific for α₁-casein (lane 3), β-casein (lane 4), and κ-caseins (lane 5). The CBB-staining patterns of SDS-PAGE for total milk proteins (lane 1) and concentrated culture medium (lane 2) are also shown for comparison. Arrowheads represent the bands immunologically detected for each casein component. Major milk proteins: α₁-casein (A), β-casein (B), κ-casein (K), β-lactoglobulin (LG), and α-lactalbumin (LA) are indicated in the left margin.
4 to 8-fold by the osmotic shock treatments. The maximum CAT activity was observed for the transfected cells with glycerol treatment for 2 min, in which about 16.4% of chloramphenicol was acetylated by the CAT assay for 60 min.

Electroporation was also used for the gene transfection of BMEC. Capacitance, voltage, and the volume of cell suspension in PBS were optimized using pSV2-CAT. An electroporation condition of 500 μFD at 400 volts (1000 V/cm) gave the maximum CAT activity. However, the CAT activity was considerably lower than that of cells transfected by the calcium phosphate method (data not shown). Hence, the calcium phosphate precipitation method in combination with glycerol osmotic shock (15%, 2 min) was used for the following transfection experiments.

III. Expression of casein-CAT chimeric genes in transfected BMEC

The milk casein-CAT chimeric genes were constructed by fusing 5′-flanking gene fragment of αs1- and β-casein amplified by PCR to CAT derived from pSV2CAT. These 5′-flanking gene segments contain several putative functional sequences, including the “milk box” and the “MGF binding sequence”, commonly found in the promoter regions of these milk protein genes.23

The plasmids containing each casein-CAT chimeric gene were transfected to BMEC by the calcium–phosphate precipitation method with the osmotic shock treatment with glycerol. As shown in Fig. 5, the CAT activity of the cells transfected with the αs1-casein-CAT chimeric gene was observed, though the activity was weaker than that of the pSV2CAT chimeric gene. The effects of the three lactogenic hormones on the expression of αs1-casein-CAT chimeric gene were also studied. BMEC were cultured in the basal medium with or without the hormones before and after gene transfection, and the CAT activity was measured as above. However, increase in the CAT activity by the hormone addition was not observed (Fig. 5). Only a slight CAT activity was detected in the cells transfected with the β-casein-CAT chimeric gene.

Discussion

To investigate introduction and transient expression of foreign genes in cultured BMEC, a primary culture of BMEC was successfully isolated with higher purity and biological functions. Although it took several days for the cells prepared as clumps to start adhering to the plastic substratum, almost no dead cells were observed in the culture, probably due to the fact that the cells exist as acinous fragments under the initial suspension stage before adhesion. These seemed to be no large differences in the cell adhesion from the acinous fragments between the plastic dishes with or without extracellular matrices (unpublished data).

Talhouk et al.9 succeeded in the preparation of cryopreserved cells as acinous fragments, and reported that the cells sustained substantial milk protein secretion when cultured on collagen substratum in serum free medium. Gibson et al.10 also isolated BMEC, which exhibited distinctive morphologic characteristics and expressed milk proteins on a collagen matrix. The BMEC reported in this
paper sustained sensitivity to lactogenic hormones, and synthesized and secreted milk caseins, even when cultivated on plastic dishes without collagen substratum. Although the adhesion of BMEC to plastic dishes coated with type I collagen or the reconstituted basement membrane from EHS tumor (Matrigel, Collaborative Research Inc.) appeared to be slightly faster than that to uncoated plastic dishes, there were no large differences in casein secretion between the cells cultured on the coated and uncoated dishes (unpublished data). Since mouse mammary cells required special extracellular matrixes for milk protein secretion, the milk protein secretion without special substrata seemed to be characteristic to bovine mammary cells, which secrete quite a large amount of milk proteins in vivo. Such property might be an advantage of BMEC for the production of valuable proteins by cultured cells. Such biological functions of BMEC in primary culture decreased gradually with increase in the number of passages, and disappeared almost completely after long-term culture. This would be the loss of differentiated biological functions of each mammary epithelial cell during the long-term culture rather than a decrease in the population of the epithelial cells, since no expansion of the contaminating fibroblast-like cells was observed throughout the cultivation. Therefore, the cells isolated from bovine mammary glands were cryopreserved in liquid nitrogen after several passages of cultivation, and used after thawing every time.

The BMEC described in this paper consist of a single large polygonal type of cells with the characteristic cobblestone morphology of epithelial cells, which form a single cell layer over the plastic substratum (Fig. 1-C, 1-D, and 1-F). Such cell type and properties are in good agreement with BMEC preparations reported by the other researchers. To introduce and transiently express foreign genes in BMEC, single cells, not clumps, with high purity in cell population are required for either calcium phosphate precipitation or the electroporation gene transfection method. Hence, the BMEC prepared in this study were suitable for efficient gene transfection.

The great increase in transfected CAT gene expression by the treatment with a DMSO or glycerol shock was first reported by Lopata et al. in mouse L cells transfected with a plasmid pSV2CAT. Malienou-Nagassa et al. reported that conventional transfection using calcium phosphate precipitation with osmotic shock of primary rabbit mammary cells markedly increased endogenous β-casein synthesis in the presence of prolactin, and that such stimulatory effects of transfection were due to transfection treatment itself but not dependent on the kinds of genes transfected. Hence, the increase in transient expression of CAT gene in transfected BMEC would also be due to the gene-independent stimulation by the glycerol shock.
treatment.

The secretion by BMEC of major milk whey proteins such as β-lactoglobulin and α-lactalbumin were not observed (Fig. 3). This might be due to lower recovery of these proteins from the culture supernatant. On the other hand, the major milk proteins, caseins, were confirmed to be synthesized and secreted by the cultured BMEC. Since the secretion of both αs1-casein and β-caseins by BMEC was demonstrated by Western blotting (Fig. 3), the expression of endogenous αs1-casein and β-casein genes was expected to be active in the cells. However, the transcription activity of a β-casein CAT chimeric gene was not detectable in the transfected BMEC (Fig. 5). This might be due to lower promoter activity of the β-casein 5′ region used rather than lower transfection efficiency of the β-casein CAT plasmid, since almost no CAT activity was also detected in BMEC transfected with CAT chimeric genes with 5′ promoter regions of the other milk proteins such as κ-casein and α-lactalbumin (unpublished data). The lower or almost no expression of β-casein CAT chimeric gene might also be explained by the results that no band corresponding to β-casein was detected in the metabolically labeled proteins secreted by BMEC (Fig. 2) and that β-casein secreted in the culture medium was markedly smaller in quantity than αs1-casein (Fig. 3). Expression of the αs1-casein CAT chimeric gene was not hormone-inducible, and the expression level was far lower than that of the SV40 CAT chimeric gene (Figs. 4 and 5). Further studies are needed to find whether the αs1-casein 5′-flanking region of about 700 bp contains no element responsible for the lactogenic hormones, or the hormone responsibility of BMEC is lost by gene transfection treatments. In any case, it could be said that although the level is still low, at least constitutive expression of αs1-casein gene was detected in the transfected BMEC. The analyses of gene segments responsible for gene expression are in progress using the homologous system using genes and cells from bovine mammary gland.

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