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Activation of the Caprine β-Lactoglobulin Gene Promoter by Lactogenic Hormones in Cultured Mammary HC11 Cells

Jaeman Kim[1, 2#], Myeong-Hee Yu[1], and Kyungjin Kim[2*]

[1]Korea Research Institute of Bioscience and Biotechnology, KIST, P. O. Box 115 Yusong, Taejeon 305-600, Korea; [2]Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea;

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Analysis of the 5'-regulatory sequence of the caprine β-lactoglobulin (BLG) gene promoter revealed that two different types of activation were mediated by discrete regions, from -740 to -470 and from -205 to 109, in cultured mammary HC11 cells. Activation mediated by the proximal region was observed regardless of cell growth status. Distal activation, however, was observed only after confluent growth of the cells and was enhanced by the lactogenic hormones. This activation was accompanied by appearance of binding activity of proteins to these regions in the mammary HC11 cells. The binding motifs were broadly distributed over the upstream regulatory sequence. Comparison of the binding regions and mutation analysis suggest that a binding motif homologous to the γ-interferon responsive element (γ-IRE) is responsible for transcriptional activation by hormonal induction in the mammary HC11 cells. The multiple γ-IRE homologous motifs seem to play a significant role in enhancing mammary cell-specific activation of the caprine BLG gene.

The mammary gland cells bear distinct features in their development: after birth, they remain unchanged until female hormones drive growth and differentiation at puberty. Activation by the lactogenic hormones during pregnancy and lactation period leads to full differentiation of the cells (Topper and Freeman, 1980). Expression of the milk-specific proteins is turned on at these stages. Expression of β-lactoglobulin (BLG), the major whey protein in ruminants' milk, also occurs in overtly differentiated mammary cells. Consequently, its expression is activated by lactogenic hormones, such as prolactin and glucocorticoid (Gaye et al., 1986; Lesueur et al., 1991; Burdon et al., 1994). The mammary tissue-specificity of the BLG gene can be depicted by the 5'-regulatory sequence (Simons et al., 1987; Wright et al., 1991; Whitelaw, 1996). Previous studies with the ovine BLG gene promoter in transgenic animals identified binding sites for the milk protein binding factor (MPBF) on its 5'-regulatory sequence (Watson et al., 1991; Burdon et al., 1994a). The binding sites were shown to mediate hormonal induction (Burdon et al., 1994a) and regulation by extracellular matrix (Streuli et al., 1995).

Transgenic studies of the ovine BLG gene also implied a possible role of the distal upstream regulatory sequence in full activation of the BLG gene. This upstream flanking sequence could enhance transcriptional activity of the proximal upstream flanking sequence of the ovine BLG promoter (Whitelaw et al., 1992). Previous studies, however, generally focused on the regulation mechanism that determines the mammary tissue-specific activation of the BLG promoter. Although the mammary tissue specificity can now be attributed to the proximal sequence, the partiality in activation of the gene left the role of the far distal upstream flanking sequence largely disregarded. Studies with transgenic animals may have bestowed some difficulties in analyzing the regulatory sequence in detail. In the present study, we adopted a cultured mammary cell line, HC11 cell, for the sake of analytical convenience. The HC11 cell was derived originally from the mammary tissue of mid-pregnant mice (Ball et al., 1988) and it responds to lactogenic hormones at full confluence without extracellular matrix (Doppler et al., 1989). The cell can represent features of both undifferentiated and differentiated mammary cells depending on the culture conditions. We analyzed the distal upstream flanking sequence for its role in activation of the caprine BLG promoter in the HC11 cells. Through the investigation,

* To whom correspondence should be addressed.
Tel: 82-2-880-6694, Fax: 82-2-872-1993
Present address; Department of Biology, Mokpo National University, 61, Torimri, Chonggye-myon, Muan-gun, Chonnam 534-729, Korea

we localized a strong activation region on the distal upstream regulatory sequence and narrowed down the most effective region required for activation. The activation region was further analyzed by an *in vitro* binding assay.

Materials and Methods

Construction of expression vectors

The 5'-regulatory sequence from -1692 to +32 of the caprine BLG gene (Kim et al., 1995) was fused to the chloramphenicol acetyltransferase (CAT) gene. The recombinant gene was inserted into the pBluescript KS(+) (Stratagene) resulting in the p1692cat expression construct. A series of deletion mutants were made by deleting progressively the distal end of regulatory sequence from the p1692cat. The 5'-regulatory sequences above -740 PstI site, -470 *SacI* site, -208 AccI site and -109 *Sma*I site were removed and named, p740cat, p470cat, p208cat and p109cat, respectively. The p205cat vector was constructed by directional deletion of nucleotides from the 5' end of the regulatory sequence of the p208cat vector with *Exo*III nuclease (Stratagene). The pcat vector was made by deleting the whole 5'-regulatory sequence from the p1692cat. Internal deletion vectors, p1692(d470)cat and p1692(d829)cat, were constructed by inserting the upstream regulatory sequences from -1692 to -470 and from -1692 to -829, respectively, in the *SacI* site of the p205cat vector. To confirm the functionality of the putative regulatory element that mediated activation, the double-stranded oligonucleotide sequence corresponding to the sequence from -744 to -697 was synthesized. The coding sequence of the synthesized oligonucleotide is 5'-GATCTAGGC AGCTCGCTGTAGCCTGAGCGTGTGGGGGAAGTGTC CTGGGAGAG-3'. In the mutant oligonucleotide, the coding sequence was changed as follows; 5'-GATCTA GGCAGCTCGAAGAGAGATGAGCGTGTGGAGGGAAGA GAGAGAGGAGAG-3'. The 5'-end and 3'-end of the oligonucleotide were made compatible to *Bgl*II and *Bam*HI sites, respectively. The wild-type oligonucleotides were inserted in forward orientation at the upstream of the regulatory sequence of the p205cat vector as a single and triplet to construct p1XOli+205cat and p3XOli+205cat, respectively. The p3XmOli+205cat vector was constructed similary to the p3XOli+205cat vector, but the wild-type oligonucleotide was replaced by the mutant oligonucleotide.

Cell culture and transfection

The CV-1 and HeLa cells were cultured in DMEM (Gibco) medium supplemented with 10% fetal bovine serum (FBS, Gibco). The HC11 (Ball et al., 1988) cells were cultured in RPMI 1640 (Gibco) containing 10% FBS and 10 ng/ml of mouse epidermal growth factor (Sigma) (Doppler et al., 1989). Culture medium was changed every two days. For transient expression assay, cells were cotransfected with 20 μg of the expression vectors and 2 μg of pCH110 (Pharmacia), a β-galactosidase expression vector, by calcium phosphate method (Ausubel et al., 1987; Sambrook et al., 1989). Two days before transfection, cells were plated at densities of 5×10^5 cells for HeLa and CV-1 cells, 2.5×10^5 cells for HC11 cells per 100 mm dish. About 3 to 7 h before transfection, cells were fed with fresh DMEM containing 10% FBS. The coprecipitates of DNA and calcium phosphate were added to the cells and incubated for 16 h. Cells were shocked by medium containing 15% glycerol. After the shock, cells were cultured for 48 h and harvested for CAT assay. Stable transformants were established by transfecting the HC11 cells with both the expression vectors and pSV2neo. Cells were cotransfected with 20 μg of the expression vectors and 2 μg of pSV2neo vector by the calcium phosphate method. Cells were selected for 10 to 15 days in growth medium containing 200 μg/ml of G418 (Gibco) starting after two days of transfection. About 100 to 200 resistant colonies were pooled and proliferated. The cells were grown to full confluence in RPMI 1640 containing 10% FBS and then stabilized in RPMI1640 containing G418 and 2% FBS for 48 h before harvest. Non-confluent cells were maintained at 10 to 30% confluence for a week and harvested.

Chloramphenicol acetyltransferase (CAT) assay

Cells were washed with PBS (Gibco) and harvested with a cell scrapper in 1ml PBS. After precipitation, the cells were resuspended in 200 μl of 0.25 M Tris buffer (pH 7.5). Cells were broken by repeated freezing and thawing 3 to 5 times. The cell extract was heat-inactivated for 10 min at 65℃ to extinguish deacylase activity (Sambrook et al., 1989). The precipitate of the heat-inactivated cell extract was removed by centrifugation and the supernatant was used to determine CAT activity or to quantitate the total protein (Ausubel et al., 1987). The CAT activity was measured by a thin layer chromatography method (Gorman et al., 1982). Each measurement was normalized to the quantity of total proteins in stable expression or to the β-galactosidase activity in transient expression (Sambrook et al., 1989).

Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared according to the procedure described (Ausubel et al., 1987). All procedures were performed at 4℃. The harvested cells were allowed to swell in hypotonic buffer on ice and disrupted in a Dounce homogenizer by 30 to 50 strokes of a B pestle. After confirming over 90% cell disruption by Trypan Blue staining, the nuclei were pelleted. The nuclei were extracted with high salt buffer containing 300 mM KCl. The extract was dialyzed in dialysis buffer for 1 h and the precipitate was removed by centrifugation. The supernatant of the extract was

aliquoted and stored in a -70℃ freezer. Protein content was determined by the Bradford method. For EMSA, 2 μg of nuclear extract in 6 μl dialysis buffer was mixed with 1 μl of 2 μg poly (dI:dC) and 1 μl of 3 mg/ml BSA on ice. The mixture was placed at room temperature for 5 min and then added with 2 μl of probe (20,000 cpm). For competition assay, about 100-fold, in molar ratio, of competitors were mixed just before adding the probes. The final volume was adjusted to 15 ml. The binding mixture was incubated at room temperature for 20 min. The mixture was separated on non-denaturing gel of 4% polyacrylamide at 4℃. After electrophoresis, the gel was dried and subjected to autoradiography.

Results

Activation of the caprine BLG promoter in cultured mammary cells

In the HC11 cells, the caprine BLG promoter downstream of -109 did not show any noticeable activity, as with the promoterless pcat vector (Fig. 1). Inclusion of the sequence from -109 to -205, however, evoked remarkable activation of the BLG promoter. The influences of the farther upstream sequences on promoter activity showed an inverse tendency depending on the states of cell growth. In non-confluent cells, sequential elongation of the upper sequence resulted in gradual reduction of the promoter activity. In confluent cells, on the contrary, the caprine BLG promoter activity was strongly enhanced once again by the -470 to -740 sequence, even without hormonal induction. This enhanced activity was slightly suppressed by inclusion of the upper sequences. For hormonal induction, cells were cultured in the medium supplemented with prolactin (5 μg/ml), dexamethasone (5 μg/ml), and insulin (5 μg/ml). These lactogenic hormones apparently intensified the activation by the upstream regulatory sequence in confluent cells. Hormonal induction was observed when the sequence above the proximal activation region, from -109 to -205, was involved. The most effective induction was mediated by the distal activation region, from -470 to -740. The role of the distal region in promoter activation was confirmed by internal deletion vectors (Fig. 1). Excision of the sequence from -470 to -208 out of the 1692 bp regulatory sequence vector did not abate the distal activation in p1692(d470)cat vector. Enlarged deletion of the region, from -829 to -208, completely abolished the distal activation. This internal deletion, however, did not suppress the repressive activity in non-confluent cells.

Characterization of the distal activation by in vitro binding assay

The distal activation region from -470 to -740 was apparently recognized by a binding factor in mammary

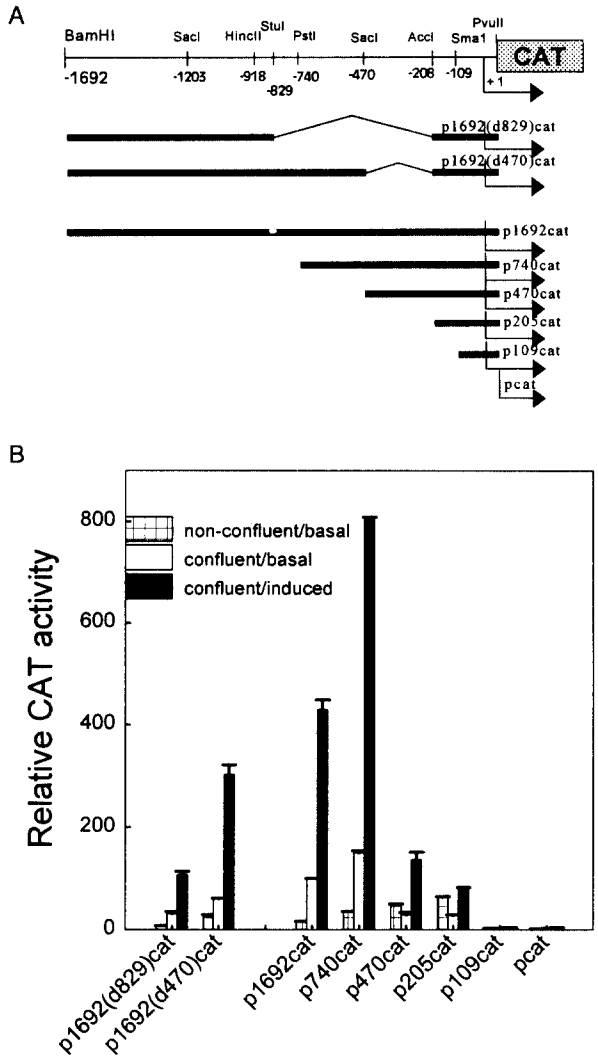

Fig. 1. Deletion analysis of the BLG 5'-regulatory sequence in stable transformed HC11 cells. A, Deletion mutant constructs. B, Relative CAT activity. The confluent cells were grown to full confluence in RPMI1640 medium supplemented with 10% fetal bovine serum and 10 ng/ml of mouse epidermal growth factor. The cells were stabilized in a medium containing 2% fetal bovine serum for 48 h. The cells were further maintained for 4 days in the same medium without (basal) or with lactogenic hormones (induced). For hormonal induction, the cells were cultured in the medium supplemented with prolactin (5 μg/ml), dexamethasone (0.1 μM) and insulin (5 μg/ml). Data are expressed mean ±SE of triplicate experiments.

cells. Such binding activity in HC 11 cells was also dependent on the state of cell growth (Fig. 2). When subjected to electrophoresis mobility shift assay (EMSA), the distal region from -663 to -740 was not recognized by the nuclear extracts from non-confluent or subconfluent HC11 cells. As the cells grew confluent, the binding activity appeared without hormonal induction. Lactogenic hormones intensified the binding activity in HC11 cells. The binding activity was not detected in non-mammary CV-1 cells. In the non-mammary HeLa cells, by contrast, a complex of compatible mobility was detected.

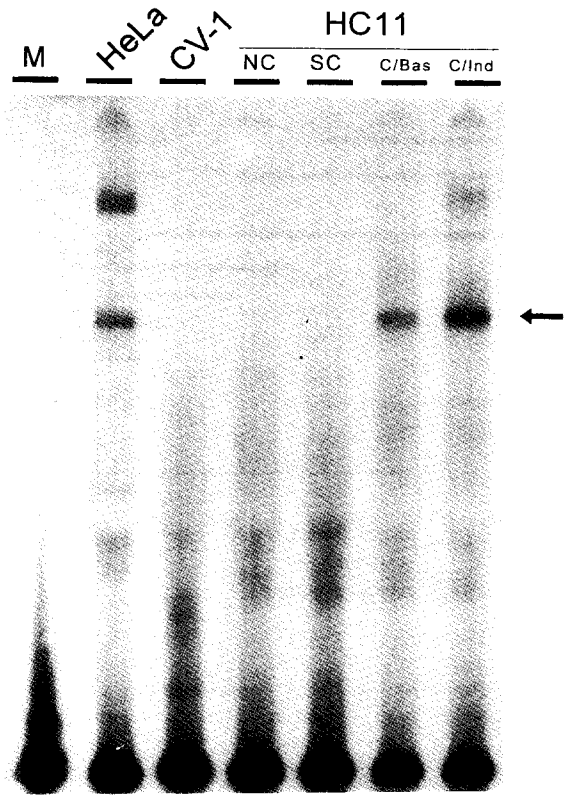

Fig. 2. Electrophoretic mobility shift assay for the putative binding factors on the sequence from -740 to -663. The nuclear extract of HC11 cells were prepared from the cells at four different growth status; NC: non-confluent, SC: sub-confluent, C/Bas: confluent and not induced, C/Ind: confluent and induced with lactogenic hormones as in Fig. 1. M indicates the lane for mock binding mixture. The arrow marks a putative activation complex.

The binding site for the complex in the HC11 cells was not restricted just to the sequence from -663 to -740. Various regions in the 5'-regulatory sequence could interfere with the binding in differing intensities (Fig. 3). Relatively strong competition was accomplished by the sequences, from -470 to -740 and from -918 to -1090. The regions from -365 to -470 and from -1090 to -1201 revealed no competition. Analysis of the competing sequences with a signal scanning program (Prestridge, 1991) suggested a most likely binding motif homologous to the γ-interferon responsive element (γ-IRE; Yang et al., 1990).

Mammary cell-specific activation with synthetic oligonucleotide

The synthesized oligonucleotide corresponding to the sequence from -744 to -697, which includes two γ-IRE homologous motifs, was heavily retarded by the nuclear extract from confluent HC11 cells (lane N in Fig. 4a). This binding was competed completely with 100-fold (100 X) wild type oligonucleotide. A mutant oligonucleotide, with the two γ-IRE homologous motifs destroyed, had its competitiveness reduced to one-fifth

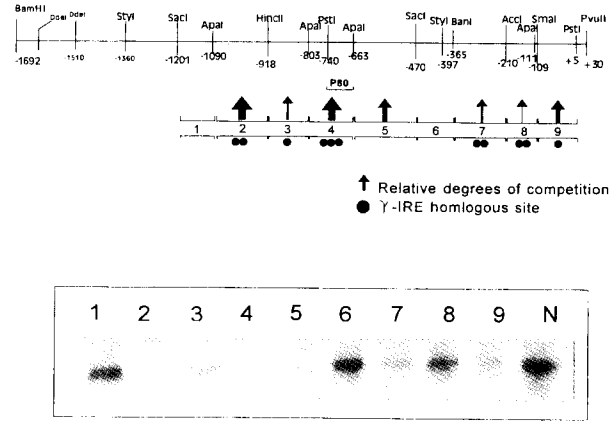

Fig. 3. Summary of competition analysis for the putative activation complex. The complex was formed between the sequence from -740 to -663 and nuclear extracts from the HC11 cells treated with lactogenic hormones. The complex formation was competed with 100-fold in molar ratio of various DNA fragments from the 5'-regulatory sequence. The numbers on each lane represent competitor fragments. N marks the lane with no competitor. P80 indicates the location of the 80 bp probe tested. The competition is summarized on the restriction map along with the number and location of γ-IRE homologous motifs, represented by the closed circles. Relative thickness of the arrows represents relative strength of the competition.

of the wild type (Fig. 4a). The synthetic oligonucleotide reserved not only the binding ability but also the transcriptional activity of the distal activation region, from -470 to -740. When the oligonucleotides were inserted in triplicates upstream of the 205 bp BLG promoter, they elevated the BLG promoter activity about 5 fold in the HC11 cells (Fig. 4b). The same construction also mediated a strong induction of the lactogenic hormones. With mutation of both the γ-IRE homologous motifs, however, transcriptional activation as well as hormonal induction disappeared. The single oligonucleotide was unable to activate downstream BLG promoter nor mediate hormonal induction. In non-mammary CV-1 and HeLa cells, the synthesized oligonucleotide did not exhibit any noticeable effect on the activity of BLG promoter. Only the HeLa cells, where a strong binding activity on the oligonucleotide was detected, displayed a trace of activation.

Discussion

A deletion analysis of the 5'-flanking sequence of the caprine BLG gene promoter in cultured mammary HC11 cells revealed two activation regions, from -109 to -205 and from -470 to -740. These two regions seem to mediate different types of activation. Activation by the proximal region was observed in both non-confluent and confluent mammary cells. This activation was not restricted to mammary cells. The activation seemed to initiate BLG promoter activity, regardless of cell state or cell type. Except in mammary cells at full differentiation such universal activation will need to be repressed, otherwise, this activation may cause inadequate expression of the BLG in non-mammary tissues

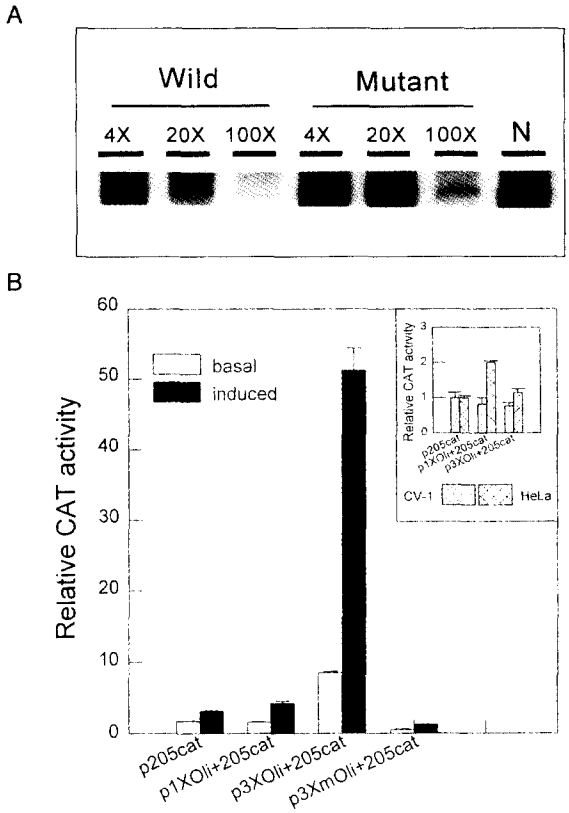

Fig. 4. Activation of the BLG promoter by the synthesized oligonucleotide in mammary cells. The synthesized oligonucleotide, corresponding to the sequence from -744 to -697 and containing two γ-IRE homologous motifs, was strongly retarded by the nuclear extract from confluent HC11 cells (lane N, N means no competition). A, The binding was competed with 4-fold (4X), 20-fold (20X) and 100-fold (100X) of oligonucleotides with intact (wild) or mutated (mutant) γ-IRE homologous motifs. To evaluate transcriptional activity of the γ-IRE homologous motifs, the synthesized wild type and mutant oligonucleotide DNA were inserted on upstream of the 205 bp promoter as a single (p1XOli+205cat) or triple (p3XOli+205cat/p3XmOli+205cat). B, The constructions were expressed in HC11 cells or in HeLa and CV-1 cells (inset). The conditions for hormonal induction were the same as described in Fig. 1. Experiments were repeated several times and data are the mean ±SE of two independent experiments of duplicates.

or in mammary cells before milk secretion. Residual suppressive activity by the upper regulatory sequence in HC11 cells seems to reflect such negative regulation in mammary cells. Because the HC11 cells are derived from mid-pregnant mammary tissue (Ball et al., 1988), they seem to reserve some features of the undifferentiated tissue which are more evident prior to confluent growth. These observations imply another regulatory mechanism underlying the mammary tissue-specificity.

Activation mediated by the distal region appeared only after the cells grew confluent. Moreover, induction with lactogenic hormones predominantly enhanced such activation. These features reconcile with activation of the BLG promoter in overtly differentiated mammary tissue, and thus seem to be related to the mammary tissue-specific activation of the BLG promoter. This type of activation was accompanied by the appearance of a binding activity in the mammary HC11 cells. Distribution of the binding motifs, however, was not limited to the distal region from -470 to -740. Even the proximal region contained the binding motif and mediated hormonal response. The dispersed distribution of the binding motifs implies the significance of the distal upstream sequence in full activation of the BLG promoter

Comparison of the sequences (Fig. 3) and mutation analysis (Fig. 5) indicated that the γ-IRE (CA/TG/TG/T ANNC/T; Yang et al., 1990) homologous motif is a putative activation element. This binding motif resembles MPBF sites (Watson et al, 1991) on the proximal regulatory sequence of the ovine BLG promoter as it activates BLG promoter in mammary cells and mediates hormonal responses. The γ-IRE homologous motif, however, was distinguished from MPBF sites in that the binding factor recognizing the motif appeared when the mammary cells grew confluent even without prolactin induction. Like the MGF binding sites (Schmitt-Ney et al., 1992; Mercier & Groner, 1994), the γ-IRE homologous motif was also recognized by a binding factor in non-mammary HeLa cells. Although the MPBF sites are related to the interferon-gamma activation sites (GAS; Burdon et al., 1994b), the γ-IRE homologous motif may be a completely different mammary cell-specific activation motif.

Activation mediated by the γ-IRE homologous motif seems to require multiple binding motifs. Although the synthetic oligonucleotide corresponding to -740 to -697 could mediate mammary cell-specific activation in triples, it could not activate the downstream promoter as a single. Existence of multiple binding motifs on the upstream regulatory sequence of the caprine BLG promoter may reflect such requirement. The present results suggest the possibility of developing an efficient expression vector that is specifically activated in mammary cells. The p3XOli+205cat vector could reproduce a specific activation and hormonal response of the p740cat vector without the intervening sequence. Adequate combination of the activation regions may greatly enhance its ability of mammary cell-specific activation. Elucidation of the activation mechanism will help the construction of a more efficient expression vector.

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