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Differential Regulation of the Caprine β-Lactoglobulin Gene Promoter in the Cultured Mammary HC11 Cells

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The β-Lactoglobulin (BLG) gene expression is differentially regulated during development of the mammary tissues. Such differential regulation of the BLG gene expression can be reiterated in the cultured mammary HC11 cells. In the growing non-confluent HC11 cells, the BLG promoter activity was shown to be partially repressed by the upstream regulatory sequence. The repression was gradually diminished and switched to activation as the cells grew confluent. The differential regulation of the BLG promoter was controlled by the 5′-regulatory sequence located at the upstream of 205 bp. Electromobility shift assay showed that nuclear extract from HC11 cells differentially bound on the regulatory sequence, depending on the cell confluency, which was in accordance with the differential transcriptional activity. DNase I foot-print assay, however, revealed that all nuclear extracts presented the same foot-prints, regardless of confluency of HC11 cells. These results suggest that differential regulation BLG gene expression by the 5′-regulatory sequence may be accomplished by competitive and/or cooperative binding of differential regulatory factors on the same regulatory element.

Mammary gland is a very specialized reproductive organ, which develops discontinuously in its progress from embryonic to lactating state during ontogeny (Topper and Freeman, 1980). After birth, there is little duct growth until the onset of reproductive cycle in a female. As the regular ovarian cycle begins, a phase of active mammary growth starts: The ducts grow and branch, and the lobular system develops. Gonadal estrogen mainly regulates growth of the mammary gland at this stage although involvement of adrenal glucocorticoids and somatotropin may also has been suggested (Hadley, 1984). Full growth of the mammary gland is achieved at the end of pregnancy or even at early lactation. Lactogenesis or milk production occurs only in overtly differentiated mammary gland at near parturition. After weaning, the mammary tissue undergoes involution and, in turn, lactogenesis stops (Delouis and Richard, 1993). Expression of the milk-specific proteins, for instance, the major whey protein in ruminants milk, β-Lactoglobulin (BLG), accords with the differentiation of the mammary tissues. In the ovine mammary tissues, its expression starts at about 2/3 of gestation period and increases sharply after delivery (Gay et al., 1986; Harris et al., 1991).

In the previous studies, it has been reported that the mammary tissue-specificity of the BLG gene expression can be depicted by the 5′-regulatory sequence alone in overtly differentiated mammary cells (Simons et al., 1987; Wright et al., 1991; Whitelaw, 1996). The present study investigated the pattern of differential regulation of the BLG promoter activity in the cultured mammary cells, HC11 cells which were derived originally from the mammary tissue of mid-pregnant mice (Ball et al., 1988). Since non-confluent HC11 cells did not respond to lactogenic hormones, while the confluent cells did (Doppler et al., 1989), it was expected that the cells might possess both undifferentiated and differentiated features according to the cell confluency. Virtually, the cells could represent both features depending on the culture condition. Relying on such special features of HC11 cells, the present study attempted to localize the region on the distal upstream regulatory sequence that is responsible for differential regulation of the caprine BLG promoter. The differential regulation was correlated to the protein binding activities to the 5′-regulatory sequence by *in vitro* electromobility shift 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) to construct the p1692cat expression vector.

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A series of deletion vectors were made by deleting progressively the distal end of regulatory sequence from the p1692cat vector. The 5′-regulatory sequence above -740 *PstI* site, -470 *SacI* site, -208 *AccI* site, and -109 *SmaI* site was removed and the expression vectors, p740cat, p470cat, p208cat and p109cat were constructed, respectively. The p205cat vector was constructed by directional deletion of nucleotides from the 5′-end of the regulatory sequence of the p208cat vector with ExoIII nuclease (Stratagene). The pcat vector was made by deleting the whole 5′-regulatory sequence from the p1692cat.

Cell culture and transfection assay

The HC11 cells (Ball et al., 1988) were cultured in the growth medium, RPMI 1640 (Gibco), containing 10% fetal bovine serum (FBS, Gibco) and 10 ng/ml of mouse epidermal growth factor (EGF, 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 5×10^4 cells for non-confluent culture and of 2.5×10^5 cells for confluent culture per 100 mm dish, respectively. About 3 to 7 h before transfection, cells were fed with fresh D-MEM containing 10% fetal bovine serum. The coprecipitates of the expression vectors and calcium phosphate were added to the medium over the cells and incubated for 16 h. Cells were shocked in 15% glycerol medium. After the glycerol shock, cells were cultured for 48 h and harvested for CAT activity assay. Stable transformants of HC11 cells were made by transfecting the cells with both the expression vectors and pSV2neo, a selection vector. Cells were cotransfected with 20 μg of the expression vectors and 2 μg of pSV2neo vector by using the calcium phosphate method. After two days culture in growth medium, cells were selected for 10 to 15 days in growth medium containing 200 μg/ml of G418 (Gibco). When the resistant colonies grew, about 100 to 200 colonies were pooled and proliferated. The non-confluent cells were grown in growth medium and subcultured for a week to keep non-confluent and harvested at 10 or 50% confluence. The confluent cells were grown to full confluence in growth medium and then stabilized in minimal medium, RPMI 1640 containing G418 and 2% fetal bovine serum for 48 h before harvest. For hormonal induction, the cells were cultured in the medium supplemented with prolactin (5 μg/ml), dexametasone (0.1 μM) and insulin (5 μg/ml).

CAT assay

The cells were washed with PBS and harvested with a cell scrapper in 1 ml PBS. After centrifugation, the pelleted 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 the assay, 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 volume of extract diluted in dialysis buffer was increased to 9 μl and the final volume was adjusted to 15 μl. 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.

Foot printing assay

The nuclear extract of about 20 to 120 μg protein in 30 μl dialysis buffer was mixed with 0.5 μg poly d (I:C), 15 μg BSA and distilled water to the final volume of 50 μl. The labeled probe of about 20,000 cpm in 2 μl was added to the mixture and incubated for 20 min at room temperature (25℃). Then 50 μl of DNase I reaction buffer containing 10mM Ca^{2+} and 5 mM Mg^{2+} was added. The mixture was digested by adding 2 μl of DNase I (Boeringer-Mannheim) dilutions of 0.1 U to 2 U for 1 minute at room temperature. DNase I digestion was stopped by adding 200 μl of reaction stopper (For 1 ml stopper, 10% SDS, 5 M NaCl, 0.5 M EDTA and 100 μg tRNA). Resulting samples were extracted with phenol-chloroform (1:1) mixture and precipitated with

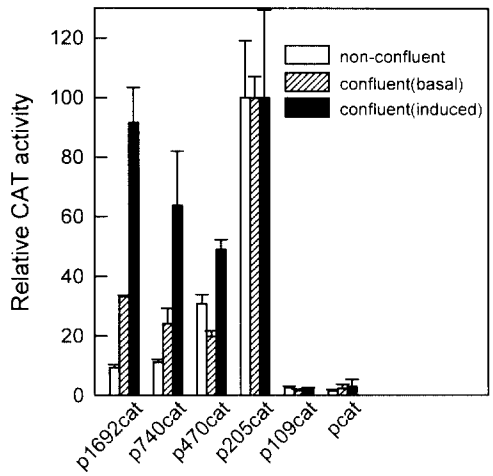

Fig. 1. The caprine BLG promoter activity in mammary HC11 cells in transient expression assay. Strategies for constructions are described in Materials and Methods in detail. The cells were plated to reach the indicated density at harvest, as described in Meterials and Methods. The transfected cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and harvested after 48 h. For hormonal induction, the cells were cultured in the medium supplemented with prolactin (5 μg/ml), dexametasone (0.1 μM) and insulin (5 μg/ml). Data shown are mean ± SE of three independent experiments.

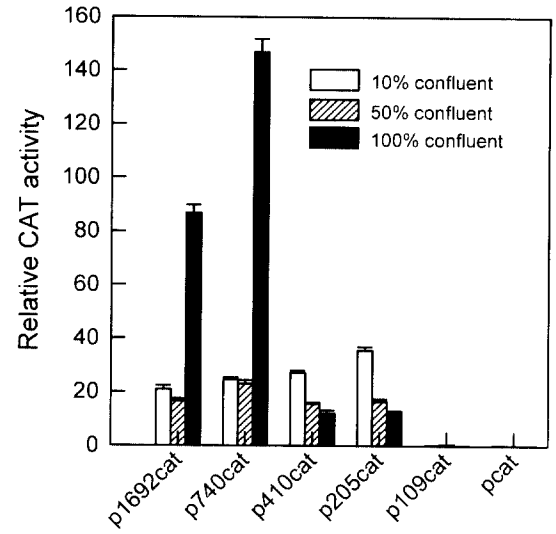

Fig. 2. Deletion analysis of 5′-regulatory sequence of the BLG promoter in stable transformed HC11 cells. The non-confluent cells were maintained a week at 10 to 30% density of confluency in the medium containing 10% FBS and 10 ng/ml EGF and then harvested at 10% and 50% cell density, respectively. The 100% confluent cells were grown to full confluence and stabilized in a medium containing 2% fetal bovine serum for 48 h. Then, the cells were harvested after further in cubation for 4 d in the same medium with (induced) or without (basal) lactogenic hormones as in Fig. 1. Experiments were repeated several times and data shown are averages of triplicates ± SE.

ethanol. The precipitate was dissolved in a loading buffer (95% formamide, etc.). As sequence references, +T and C reaction of Maxam-Gilbert sequencing was performed according to the suppliers manual (Sigma). The DNase I reaction samples were separated on 6% urea-polyacrylamide gel.

Results

Effect of culture conditions on activation of the caprine BLG promoter

Regulation of the caprine BLG promoter activity was influenced by the growth state of the HC11 cells and configuration of the 5′-regulatory elements in the cells. The proximal upstream regulatory sequence from -109 to -205 significantly activated downstream BLG promoter regardless of cell density in a transient expression assay (Fig. 1). This enhanced transcription was suppressed by the distal upstream regulatory sequence in the non-confluent HC11 cells. Confluent growth and hormonal induction diminished the repression progressively. Effects of cell growth and hormonal treatment were clearly seen in the stable transformants (Fig. 2). In the cells at 10% confluence, the 5′-regulatory sequence above -205 slightly repressed the downstream BLG promoter activity. As the cells grew to 50% confluence, such repression almost disappeared and weak activation by lactogenic hormones began to reveal, especially by the region from -410 to -740. Confluent growth replaced the minor repression with strong activation by the same regulatory sequence. The sequence below -205, however, did not discriminate the cell states in activating downstream promoter activity. The basal promoter region below -109 did not show any appreciable transcription activity. Effect of hormonal induction was amplified as the culture maintained for a prolonged period. During the culture, the stable transformants of HC11 cells at full confluence could be maintained, at least, for 2 wk without loss of cell viability in the minimal media, RPMI 1640 containing 2% FBS. The level of basal expression remained almost stable through the culture period. The BLG promoter activity under hormonal induction, however, increased steadily up to 5 folds and 9 folds in p1692 cat and p740cat vector, respectively (Fig. 3).

Differential binding activity on the 5′-regulatory sequence of BLG gene

The distal regulatory region above -205 revealed the differential binding complexes in accordance with its transcriptional activity (Fig. 4). When the region from -663 to -740 was subjected to EMSA, it was not recognized by the nuclear extracts from non-confluent HC11 cells. The extract from the confluent cells formed a binding complex without hormonal induction. Lactogenic hormones intensified the binding activity in HC11 cells. The lower regions, from -663 to -470, from -470 to -365, from -365 to -208, also exhibited similar binding patterns. In particular, the regions from -663 to -470 and from -365 to -208 represented a noble binding complex in response to hormonal induction. Moreover, the two regions, from -470 to -365 and from -365 to -208, revealed another fast-moving complex. This com-

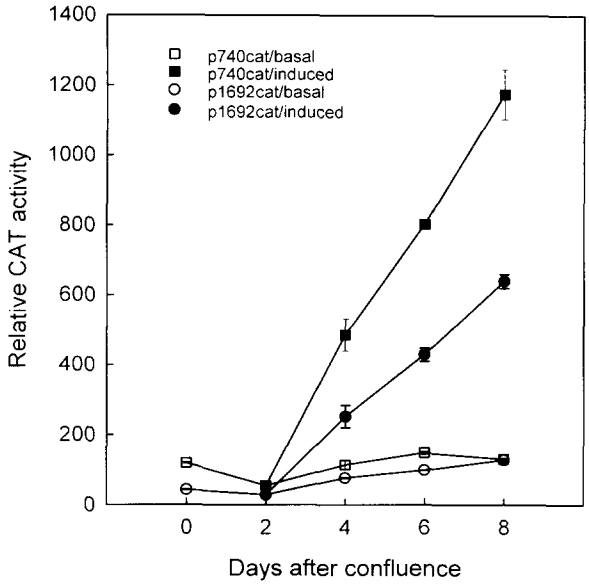

Fig. 3. Hormonal activation of the caprine BLG promoter activity in prolonged culture. The stable transformants transfected with p1692cat and p740cat vector were grown to full confluence in RPMI 1640 media supplemented with 10% FBS and 10 ng/ml of epidermal growth factor. When the cells grew confluent, the time was set up as day 0 of confluence. The cells were stabilized in 2% FBS media for 48 h and were committed to prolonged culture with (closed symbols) or without hormonal induction (open symbols). Hormonal induction was carried out as described in Fig. 1. Data shown are averages of duplicates ± SE.

plex was slightly weakened inversely with the slow-moving complexes. To identify the regulatory elements which were responsible for the differential regulation and differential binding, the sequence from -208 to -470, which formed three types of complexes in the hormone-induced HC11 cells, was protected with nuclear extracts from HC11 cells at different growth states and subjected to DNase I digestion. In the analysis, at

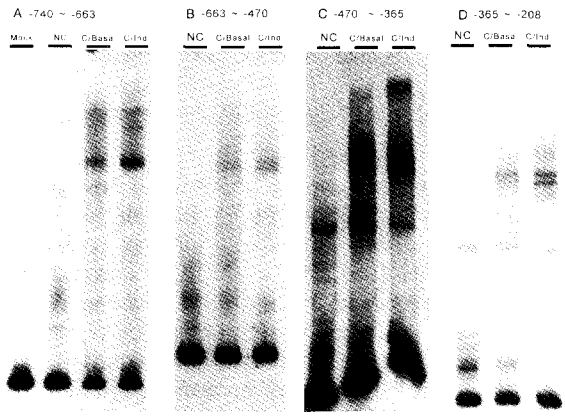

Fig. 4. Electrophoretic mobility shift assay for the putative activation and repression elements. The upstream regulatory sequences, from -740 to -663 (A), from -663 to -470 (B), from -470 to -365 (C), and from -365 to -208 (D), were subjected to electrophoretic mobility shift assay. The nuclear extract of HC11 cells was prepared from the cells at three different growth states; NC: non-confluent, C/Basal: Confluent and basal, C/Ind.: confluent and induced with lactogenic hormones as in Fig. 1. M indicates the lane for mock binding mixture.

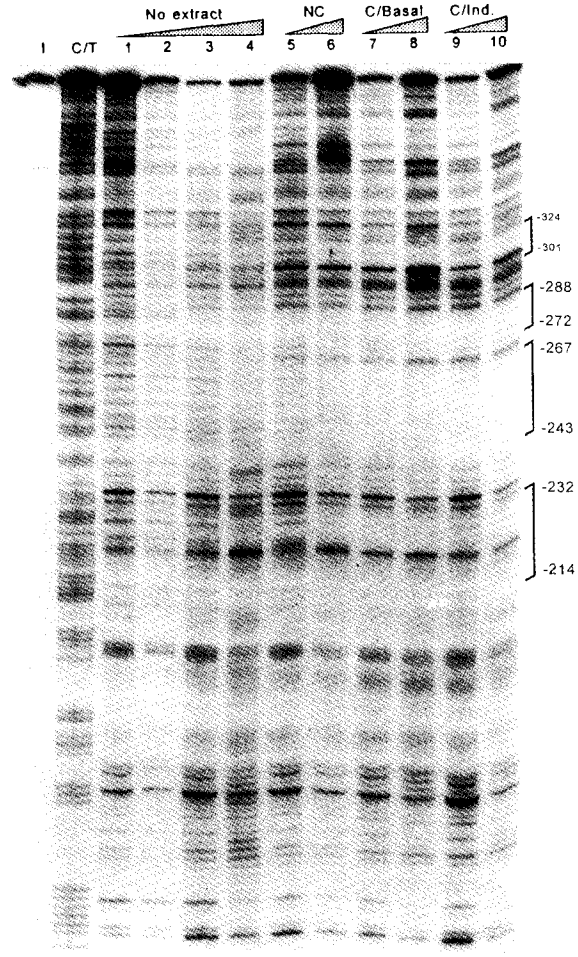

Fig. 5. *In vitro* footprinting of the 5′-regulatory sequence from -470 to -205. The probe DNA (-470 to -109 region) was reacted with HC11 extracts prepared from NC, C/basal and C/Ind. states as described in Fig. 4 and then digested with DNase I enzyme. Four major protected regions are indicated on at the right side of Fig. 1; Intact probe, C/T and C; C/T reaction and C reaction in Maxam-Gilbert sequencing. The lanes numbered are the samples digested with DNase I in the presence of (5 to 10) or in the absence of nuclear extract (1 to 4). The wedge on the top indicates the increasing amount of extract (in protected digestion) or DNase I (in unprotected digestion).

least four regions, from -214 to -232, from -243 to -267, from -272 to -288 and, from -301 to -324, were protected from DNase I digestion (Fig. 5). Among the four protected regions, the two regions from -272 to -288 and -243 to -267 were protected more preferentially than the other two regions. The sequence homology between the protected regions was not recognizable. It was noticeable that no apparent difference was observed between the extracts which displayed different complex formation patterns in EMSA.

Discussion

Differential regulation of the BLG promoter activity is an essential feature of the gene because its expression should be turned on and off through the cyclic development of the mammary tissue. Such rationale is com-

plicated by the constitutive activity of the proximal region (Figs. 1 and 2). In the present study, I found such complex differential regulation in the cultured mammary HC11 cells. The cells at non-confluent culture grew and divided actively under the influence of epidermal growth factor. In this condition, lactogenic hormones hardly induced milk gene activity (Doppler et al., 1989; Hynes et al., 1990). As the cells grew confluent, cell division and growth almost ceased. Treatment with lactogenic hormones at this condition seemed to promote protein synthesis (data not shown). Cell division, however, did not resume with hormonal induction. The caprine BLG promoter revealed differential regulation patterns in the cultured HC11 cells at different culture conditions. Because the proximal activation region from -205 to -109 activates downstream promoter constitutively, the obligation for differential regulation must be owed to the upper regulatory sequence. In transient expression assay, repression by the distal upstream sequence was exhibited obviously. Confluent growth and hormone treatment largely alleviated the repression in the presence of the distal upstream sequence. The role of the distal regulatory sequence after confluent grow was clearly seen through the stable expression assay. The regulatory sequence above -410 significantly elevated BLG promoter activity even without hormonal induction. Hormonal induction intensified such confluence-dependent activation. Insufficient activation in transient expression assay may suggest requirement of chromosomal integration for full activation of the BLG promoter. Differential regulation of the promoter activity between transient and stable expression, which was influenced by its configuration in the cell, was previously reported with MMTV promoter (Archer et al., 1992). Shortened culture time after confluence in transient expression may be another reason for reduced activation.

Differential regulation in the HC11 cells at different culture conditions was in harmony with appearance of binding activities in the cells. The sequence above -205 was recognized by differential binding activities. The sequence was complexed by the binding factor which was intensified with confluent growth and hormonal induction. Hormonal induction disclosed another binding complex on the sequences within the regions from -205 to -663. These complexes seem to be responsible for promoter activation, The region from -205 to -410 formed the third complex which was weakened with confluent growth and hormonal induction on the contrary to the former complex. This complex had similar mobility and competition tendency to the complex in the non-mammary cells (manuscript submitted). The complex seems to be related to repression of the BLG promoter. This complex, however, endured prolonged culture in HC11 cells under hormonal treatment (unpublished). This observation may give an explanation for the resisting repression by the far upstream sequence above -740 in the hormone-induced culture. Differential binding complexes in the *in vitro* binding assay, on the other hand, did not result in differential foot-printings (Fig. 5). In spite of obvious differences in the binding complexes between the extracts from different growth, all the extracts presented the same foot prints. The results suggest that differential regulation in the HC11 cells may not originate from binding on different regulatory elements, but may arise from modification in binding on the same binding element. The modification may include replacement of the binding factors and/or reorganization of the binding complexes. The exact mechanism of the active promoter regulation by binding factors and the identity of the binding factors shall be elucidated by future investigation.

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