Korean J. Zool. 38:426-432, 1995

Hormonal Regulation of the Caprine β-Lactoglobulin Gene Promoter Activity

Jaeman Kim[1,2] and Kyungjin Kim[2]

[1]Korea Research Institute of Bioscience and Biotechnology, KIST, Taejeon 305-333; [2]Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Expression of β-lactoglobulin gene in mammary tissue is strongly induced by lactogenic hormones such as prolactin, glucocorticoid, and insulin. In order to elucidate the regulatory mechanism underlying such hormonal induction, the response of the caprine β-lactoglobulin gene promoter to lactogenic hormones was analyzed in cultured HC11 mammary cells. Expression with serial deletions of the 5′-regulatory sequence of the β-lactoglobulin promoter revealed that two regions are responsible for a substantial change in hormonal inducibility. The region upstream of −1692, which exhibited strong repression of the downstream promoter, mediated the induction by insulin. This insulin-response was independent of the other two lactogenic hormones, prolactin and glucocorticoid. The other region from −740 to −470, which showed strong activation of the β-lactoglobulin promoter in confluent HC11 mammary cells, mediated mainly the response to a glucocorticoid analogue, dexametasone. The induction by the latter region, however, was suppressed by the usptream repression without insulin treatment. These results suggest that the induction of β-lactoglobulin promoter activity by lactogenic hormones in mammary cells may be achieved by the combined action of derepression by in sulin and activation by glucocorticoid and prolactin. Dexametasone response by the latter region seems to be mediated by the glucocorticoid receptor site around −700bp.

KEY WORDS: Caprine β-Lactoglobulin Promoter, Lactogenic Hormones, Insulin, Prolactin, Glucocorticoid Receptor Site

Gene expression of milk-specific proteins is precisely controlled according to the schedule of mammary gland development. The genes for milk-specific proteins remain inactive while the mammary tissue grows and differentiates before gestation. At pregnancy, the mammary tissue undergoes further differentiation under the influence of the various hormones and growth factors (Topper and Freeman, 1980; Cowie, 1984). Expression of milk-specific genes is onset during gestation by appropriate signals in the overtly differentiated mammary cells. Lactogenic hormones such as prolactin, glucocorticoid, and insulin are known to play a major role in regulation of the milk-specific gene expression (Cowie, 1984).

Determination of temporal and spatial specificity in expression of milk specific genes seems to be mediated by the regulatory sequence flanking the genes. Expression of the β-lactoglobulin (BLG), one of the milk-specific proteins, is also regulated in a mammary tissue-specific manner by the 5′-regulatory sequence (Whitelaw *et al.*, 1992; Wright *et al.*, 1991). In

the previous study (Kim *et al.*, 1995), we isolated the caprine BLG gene from the caprine genomic library and showed that the 5′-regulatory sequence of 1.7 kb can direct mammary cell-specific expression. The mammary tissue-specificity was secured by strong repression of the promoter activity with the upstream regulatory sequence in non-mammary and growing mammary cells (manuscript in preparation). In order to achieve mammary tissue-specific activation of the BLG promoter, the repression should be relieved in overtly differentiated mammary cells. Hormonal relief of repression has been reported in the expression of β-casein gene (Schmitt-Ney *et al.*, 1991). The BLG gene is also induced by such lactogenic hormones as prolactin (Lesueur *et al.*, 1991) and glucocorticoid (Gay *et al.*, 1986). In order to characterize the mechanism for hormonal induction of the BLG gene, we analyzed hormonal response of the 5′-regulatory sequence of the caprine BLG promoter in HC11 cells. Changes in hormonal inducibility by sequential truncation of the 5′-regulatory sequence showed that two regions mediate distinct hormonal response to various lactogenic hormones. A putative regulatory mechanism of the respective regions was discussed.

Materials and Methods

Construction of expression vectors

The 5′-regulatory sequence of 1.7 kb, *Bam*HI-*Pvu*II fragment from the 4.4 kb *Bam*HI fragment of the caprine BLG gene (Kim *et al.*, 1995) was fused to the chloramphenicol acetyltransferase (CAT) gene. The recombinant gene was inserted to the pBluescript KS(+) (Stratagene) to construct the p1692cat expression vector. The p5400cat vector was constructed by linking the 5′-end of the regulatory sequence of the p1692cat vector to the adjacent 3.7 kb *Bam*HI fragment (Kim *et al.*, 1995). 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 *Pst*I site, −470 *Sac*I site, −208 *Acc*I site and −109 *Sma*I site was removed and the expression vectors, p740cat, p470cat, p208cat and p109cat were constructed, respectively. The p205cat vector was constructed by directional removal of nucleotides from 5′ end of the regulatory sequence of p208cat vector with *Exo*III nuclease (Stratagene). The pcat vector was made by removing the whole 5′-regulatory sequence from the p1692cat. To construct the vectors p1XOli+205cat, p3XOli+205cat and p3XmOli+205cat, the double-stranded oligonucleotide was synthesized and inserted in forward orientation at the 5′-end of the regulatory sequence of p205cat vector as a singlet or triplet, respectively. The oligonucleotide corresponds to the sequence from -744 to -697bp of 5′-regulatory sequence of the caprine BLG gene and sequence of the coding strand is as follows. 5′-ATCTAGGCAGCTCGCTGTAGCCTGAGCGTGT GGAGGGAAGTGTCCTGGGAGAG-3′. The glucocorticoid receptor site on the oligonucleotide (underlined) mutated in the mutant oligonucleotide; 5′-GATCTAGGCAGCTCGAAGAGAGATGAGC GTGTGGAGGGAAGAGAGAGAGGAGAG-3′ The 5′-end and 3′-end the oligonucleotide were made compatible to *Bgl*II and *Bam*HI site, respectively to aid facile construction.

Cell culture

The HC11 cells were grown in the growth medium, RPMI 1640 (Gibco) medium containing 10% fetal bovine serum and 10 ng/ml mouse epidermal growth factor (Sigma; Doppler *et al.*, 1989). Culture medium was changed every two days. Stable transformants of HC11 cells were made by transfecting the cells both with expression vectors and pSV2neo, selection vector. The cells were cotransfected with 20 μg of the expression vectors and 2 μg of pSV2neo vector, by calcium phosphate method (Sambrook *et al.*, 1989). About 3 to 7 hours before transfection, the cells were fed with fresh D-MEM containing 10% fetal bovine serum. The coprecipitates of DNA of expression vectors and calcium phosphate were added to the medium over the cells and incubated for 16 hours. The cells were shocked with 15% glycerol medium and were cultured in growth medium for 48 hours. After two days growth in growth medium, the cells were selected for 10 to 15 days in selection medium, which is growth

medium containing 200 μg/ml of G418 (Gibco). When the resistant colonies were grown apparently, about 100 to 200 colonies were pooled and proliferated. The transformed cells were grown to full confluency in growth medium and stabilized in the minimal medium, RPMI1640 containing G418 and 2% fetal bovine serum for 48 hours. When the cells were induced with lactogenic hormones (Doppler *et al.*, 1989) the stabilized cells were cultured in the minimal medium supplemented with 5 μg/ml of bovine insulin (Sigma), 5 μg/ml of ovine prolactin (Sigma) and 0.1 μM of dexametasone (Sigma) for indicated times. Non-induced cells were maintained in the minimal medium.

Chloramphenicol acetyltransferase (CAT) assay

The cells were washed three times with PBS (Gibco) and added with 500 μl lysis buffer (MOPS-buffer containing NaCl and Triton X-100; Boeringer-Mannheim). After 30 minutes incubation at room temperature, the cell extract was transferred to a microfuge tube and spinned to remove precipitated debris. The supernatant cell extract was used in determining the quantity of total protein (Ausubell *et al.*, 1987) or chloramphenicol acetyltransferase (CAT) enzyme. The quantity of CAT enzyme was determined by immunological method with CAT ELISA kit (Boeringer-Mannheim). Each measurement was normalized to the quantity of total protein.

Results and Discussion

In order to localize the region that mediates the regulation of lactogenic hormones, the 5.4 kb 5′-regulatory sequence of the caprine BLG promoter was analyzed in cultured HC11 mammary cells (Fig. 1). When the stable transformants of BLG-CAT construct were treated with the three major lactogenic hormones, insulin, dexametasone, and prolactin, the expression level of the CAT enzyme increased by 8 folds. Hormonal inducibility of the 5.4 kb 5′-regulatory sequence was most sensitive to insulin. Reduction of insulin concentration sharply decreased inducibility down to almost null.

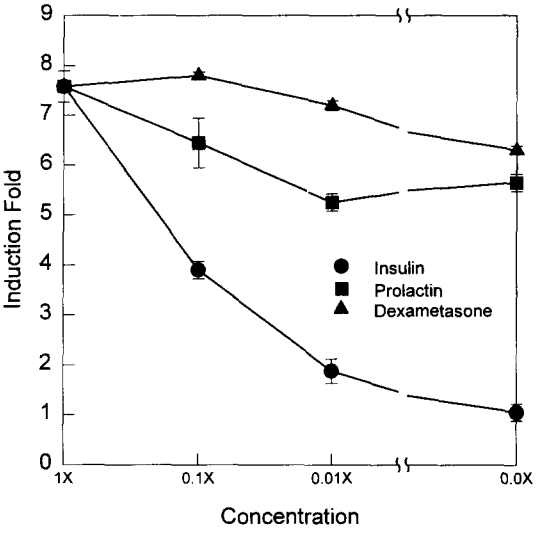

Fig. 1. Effect of lactogenic hormones on the 5.4 kb 5′-regulatory sequence of the caprine BLG gene. The stable transformants transfected with p5400cat vector were induced with lactogenic hormones, insulin, prolactin, and dexametasone. The concentration of one indicated hormone was varied, while the concentration of the other two was kept constant. The standard concentrations (1X) of each hormone are: insulin (5 μg/ml), prolactin (5 μg/ml) and dexametasone (0.1 μM).

Reduction in prolactin or dexametasone, however, did not display any profound effects. In order to identify the sequence responsible for hormonal inducibility, serial deletions of the sequence were examined (Fig. 2). Sequential deletion analysis of the caprine 5′-regulatory sequence revealed that two regions strongly mediated the induction by lactogenic hormones. Deletion of the 5′-regulatory sequence above −1692 reduced the hormonal inducibility by half. The deletion, however, increased the basal (without hormone treatments) level of expression by 10 folds, suggesting that the sequence upstream of −1692 carries negative elements. Deletion of the region from −740 to −470 also substantially decreased the hormonal inducibility. Unlike the upstream region, deletion of this region decreased the basal expression.

In order to examine the specificity in hormonal response of the two regions, responses to individual lactogenic hormone were investigated. Induction mediated by the regulatory sequence

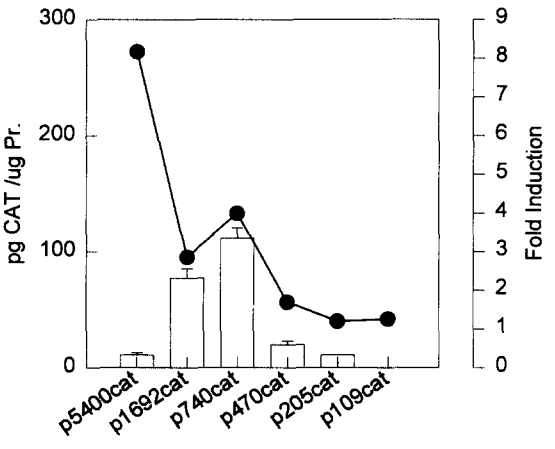

Fig. 2. Changes in hormonal inducibility by serial deletion of the 5′-regulatory sequence of the caprine BLG gene. The HC11 mammary cells were stably transfected with a series of expression vectors carrying various deletions in the 5′-regulatory sequence of the BLG promoter. Induction with lactogenic hormones was performed as described in Materials and Methods. The CAT enzyme was quantitated by ELISA method. Experiments were repeated several times and the data shown are the average of triplicates.

above −1692 was mostly dependent on insulin (Fig. 3a). The region from −1692 to −470 exhibited only a weak respone to insulin. Induction by the region from −740 to −470, however, was closely mimicked by dexametasone treatment alone (Fig. 3b). Without upstream sequence, this region increased the expression level by 2 folds. Induction by this region, however, was concealed by the upstream sequence. The observed repression pattern was consistent with that observed in the basal (without hormone treatment) expression of the BLG promoter (Fig. 2). The results suggest that dexametasone is not directly involved in the activity of the upstream sequence. Treatment with prolactin alone did not exhibited any significant activation (Fig. 3c). Combined induction by prolactin and dexametasone, however, increased the inducibility of the region above −740 (Fig. 3d). Treatment with the two hormones seemed to overcome partially the repression by the sequence from −1692 to −740, but not the repression by the sequence of further upstream. Induction by the two hormones, however, has not fully relieved the repression of the upstream sequence (Figs. 3d, 2). These results suggest strongly that the induction by glucocorticoid or prolactin should be accompanied by insulin treatment. The insulin effect was localized at the region upstream of −1692 (Fig. 3a). Localization of the regions that conferred strong repression and that mediated insulin response suggests that insulin may act through derepression. Hormonal derepression was previously reported in activation of β-casein promoter in HC11 cells (Schmitt-Ney *et al.*, 1991). Our results suggest that individual lactogenic hormone plays a distinct role in induction of the caprine BLG gene. The results also suggest that hormonal induction of the caprine BLG promoter activity may be accomplished via two distinct phases: insulin may activate BLG promoter through derepression of the region above -1692, and then the sequence from −1692 to −470bp may mediate the dexametasone and prolactin responses.

The regulatory sequence from −740 to −470 which mediated activation by dexametasone alone (Fig. 3b) contained two glucocorticoid receptor (GR) sites (Kim *et al.*, 1995). In order to confirm the functionality of the GR sites, an oligonucleotide corresponding to the sequence form −744 to −697, which involves one of the GR sites (Kim *et al.*, 1995), was synthesized. The synthesized oligonucleotide inserted as a triplet on the upstream of 205 bp BLG promoter provided dexametasone response to the construct (Fig. 4). This response, however, was not detected when the glococorticoid receptor site on the oligonucleotide was mutated. Single insert of the oligonucleotide could not mediate glococorticoid response, neither.

The dexametasone response may be achieved rather directly. Because dexametasone is an analogue of a steroid hormone, glucocorticoid, it may enter the nucleus directly after being complexed with receptor. Direct activation by the steroid hormones through specific receptor site had been reported in other milk genes. Activation of the human lactoferrin promoter with estrogen via estrogen receptor binding site (Liu and Teng, 1991) and repression of the β-casein promoter via progesterone receptor site (Lee and Oka, 1992)

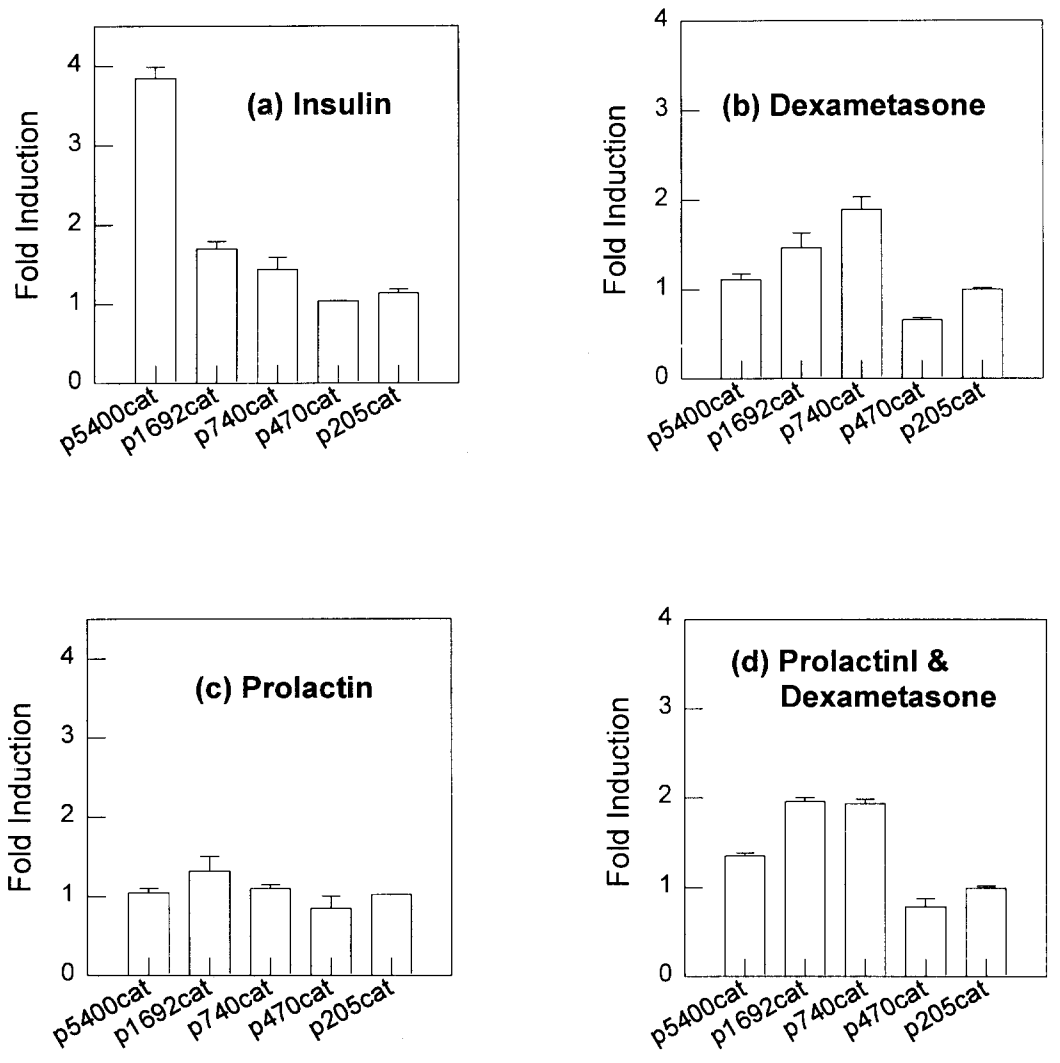

Fig. 3. Responses of various 5′-regulatory sequences of the caprine BLG gene to individual lactogenic hormones. Stable transformants of a series of deletion vectors were induced with indicated hormones. Inducibility was calculated as a ratio between the expression level induced by hormones and the basal expression level.

are the reported cases. On the caprine BLG promoter, the region that mediated dexametasone response contained two of the three clustered GR sites (Fig. 2). Moreover, the oligonucleotide with intact GR site mediated dexametasone response while the oligonucleotide with mutation in the GR site revealed no response (Fig. 4). Such agreement in occurrence of dexametasone response and the GR sites suggests that the putative GR sites around −700 may be functional in the caprine BLG gene. The fact that tripled oligonucleotide mediated dexametasone responses efficiently, while single oligonucleotide did not function suggests that functional array of GR site may be the repeated occurrence of the sites. Clustered occurrence of the GR sites supports such explanation. There is a possibility that dexametasone may work at other activation elements in the region via indirect induction of other transcriptional machinery. In confluent HC11 cells, the region that mediated dexametasone response exhibited strong activation

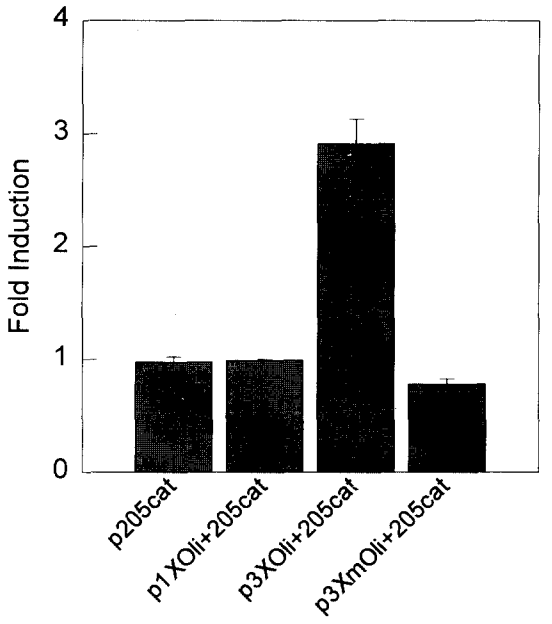

Fig. 4. Response of the seqence from -744 to -697 to a glucocorticoid analogue, dexametasone. Effect of the insertion of the oligonucleotide carrying the seqence from -744 to -697 was examined on the response to a glucocorticoid analogue, dexametasone. The HC11 cells were transfected with the p205cat vector with the 205 BLG promoter, the vectors carrying one synthetic oligonucleotide (p1XOli+205cat) or three insertions (p3XOli+205cat) fused to the basal 205 BLG promoter and the vector with three mutant oligonucleotides (p3XmOli+205cat). The stable transformants were induced with 0.1 μM dexametasone. Other experimental conditions were described in the legend of Fig. 1.

instead of repression, even without hormonal induction (Fig. 2).

In contrast to the distinct actions of insulin and dexametasone on the BLG promoter in HC11 cells, a prominent role of prolactin, the major lactogenic hormone, was not observed. There is an indication that prolactin, in conjunction with dexametasone, may relieve the repression conferred by the region from -1692 to -740 (c.f. Fig. 3b, 5d). The 4 kb 5′-regulatory sequence of the ovine BLG gene mediated strong induction in cultured CHO cells when the regulatory sequence was cotransfected with prolactin receptor gene (Lesueur *et al.*, 1991). Its effect was suggested to be mediated by milk protein binding factor (MPBF) sites (Burden *et al.*, 1994). Although the MPBF sites were conserved relatively well in the caprine gene (Kim *et al.*, 1995), it was not successful to observe any significant induction by prolactin alone in the mammary HC11 cells. If the effect of prolactin is achieved by participation in differentiation of the mammary cells, its function may be obscured in an in vitro system of partially differentiated HC11 cells.

By summing up the previous results, the following working model for hormonal induction of the caprine BLG promoter may be suggested. When the BLG promoter is activated by lactogenic hormones, the repression may be relieved by insulin. Glucocorticoid and prolactin then activate the promoter additively. Further studies are needed to approve the suggestion.

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J. G. Seidman, J.A. Smith, and K. Struhl, 1987. Quantitation of Proteins, In: Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, pp.10.1.1-10.1.3.

Burdon, T.G., K.A. Maitland, A.J. Clark, R. Wallace, and C.J. Watson, 1994. Regulation of the sheep β-lactoglobulin gene by lactogenic hormones is mediated by a transcription factor that binds an interferon-γ activation site-related element. *Mol. Endocrinol.* **8:** 1528-1536.

Cowie, A.T., 1984. Lactation, p.195-231. Reproduction in Mammals, In: Hormonal Control of Reproduction (C.R. Austin, and R.V. Short, eds.). Press Syndicate of the University of Cambridge, Cambridge, pp.195-231.

Doppler, W., B. Groner, and R.K. Ball, 1989. Prolactin and glucocorticoid hormones synergistically induce expression of transfected β-casein gene. *Proc. Natl. Acad. Sci. USA* **86:** 104-108.

Gaye, P., D. Hue-Delahaie, J.-C. Mercier, S. Soulier, J.-L. Vilotte, and J.-P. Furet, 1986. Ovine β-lactoglobulin messenger RNA: nucleotide sequence and mRNA levels during functional lactation. *Biochimie* **68:** 1097-1107.

Kim, J., J-Y. Kim, K. Kim, and M-H. Yu, 1995. Isolation and characterization of the caprine genomic β-lactoglobulin gene. *Mol. Cells* **5:** 209-216.

Lesueur, L., M. Edery, S. Ali, J. Paly, and P.A. Kelly, 1991. Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein

gene transcription. *Proc. Natl. Acad. Sci. USA* **88:** 824-828.

Liu, Y. and C.T. Teng, 1991. Characterization of estrogen-responsive mouse lactoferrin promoter. *J. Biol. Chem.* **266:** 21880-21885.

Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989. Expression of the Cloned Genes in Cultured Mammalian Cells, In: Molecular Cloning (N. Ford, C. Nolan, and M. Ferguson, eds.). Cold Spring Harbor Laboratory Press, New York, pp.16.1-16.81.

Schmitt-Ney, M., W. Doppler, R.K. Ball, and B. Groner, 1991. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. *Mol. Cell. Biol.* **11:** 3745-3755.

Topper, Y.J. and C.S. Freeman, 1980. Multiple hormone interactions in the developmental biology ofthe mammary gland. *Physiol. Rev.* **60:** 1049-1106.

Whitelaw, C.BA., S. Harris, M. McClenaghan, J.P. Simons, and A.J. Clark, 1992. Position-independent expression of the ovine β-lactoglobulin gene in transgenic mice. *Biochem. J.* **286:** 31-39.

Wright, G., A. Carver, D. Cottom, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner, and A. Colman, 1991. High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Bio/Technol.* **9:** 830-834.

(Accepted June 8, 1995)

염소의 베타-락토글로불린 유전자 프로모터 활성의 호르몬에 의한 조절

김재만[1,2], 김경진[2]([1]한국과학기술연구원 생명공학연구소, [2]서울대학교 자연대 분자생물학과)

유선 조직에서 베타-락토글로불린 유전자의 발현은 프롤락틴, 글루코코르티코이드 및 인슐린등의 유촉진 호르몬들에 의해서 강력하게 유도된다. 이와 같은 호르몬 유도의 조절 기작을 규명하기 위하여, 배양 유선 세포인 HC11 세포에서 염소 베타-락토글로불린 유전자 프로모터의 유촉진 호르몬에 대한 반응을 분석하였다. 베타-락토글로불린 프로모터의 5′-조절 부위를 연쇄적으로 제거한 발현 실험에서 호르몬 유도를 크게 변화 시키는 두 지역이 관찰되었다. 조절 부위의 −1692의 상류지역은 하류 프로모터의 활성을 크게 억제하는 동시에 인슐린 유도를 매개하였다. 이 인슐린 반응은 다른 두 유촉진 호르몬인 프롤락틴과 글루코코르티코이드와는 무관하게 나타났다. 또 다른 부위인 −740 부터 −470까지의 지역은 충만되게 자란 HC11 세포에서 베타-락토글로불린 프로모터를 강력하게 활성화 시키는 부위로, 주로 글루코코르티코이드 유도체인 덱사메타손의 작용을 매개하였다. 그러나 두번째 지역의 유도 작용은 인슐란 처리를 병행하지 않을 경우 상류 조절부위에 의해 억제되었다. 이러한 결과는, 유선세포에서 유촉진 호르몬들에 의한 베타-락토글로불린 프로모터 활성 유도가 인슐린에 의한 탈 억제화와 글루코코르티코이드 및 프롤락틴에 의한 활성화 의 복합 조절에 의해서 이루어질 것이라는 점을 시사한다. 두번째 지역에 의한 덱사메타손 유도는 −700 부근의 글루코코르티코이드 수용체 결합 부위에 의해서 매개되는 것으로 추정된다.