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Identification of the Negative Regulatory Element on the Caprine β-Lactoglobulin Promoter

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Mammary tissue-specificity of the caprine β-lactoglobulin promoter appears to be secured by repression in non-expressing cells. In order to identify the mechanism of the negative regulation, the upstream promoter sequence of the caprine β-lactoglobulin gene was analyzed in detail. The repression was mediated by the upstream flanking sequence from -470 to -205. The sequence could repress the promoter activity of β-lactoglobulin in either orientation. The effect of the putative negative regulation element of caprine β-lactoglobulin on heterlogous promoters, however, varied: the promoter activity of herpes simplex virus *thimidine kinase* was either repressed or activated by the sequence depending on its orientation, while the SV40 early promoter was activated rather than repressed. The regulatory sequence involving the putative negative regulatory element was strongly shifted with the nuclear extract from non-mammary HeLa and CV-1 cells, while only weak shift was observed with that of mammary HC11 cells. Such correlation between repression and factor binding suggests that the protected regions in foot-printing assay may be the negative regulatory elements of β-lactoglobulin that serve tissue-specific repression.

KEY WORDS: Negative Regulatory Element, Caprine β-Lactoglobulin Promoter, Tissue-specificity

Beta-lactoglobulin (BLG) is one of the milk specific proteins expressed in fully differentiated mammary tissue (Harris *et al.*, 1990). Expression of BLG is detected only in the mammary tissue of the pregnant or lactating individual (Whitelaw *et al.*, 1992). Mammary tissue-specificity of the BLG gene appears to be accomplished primarily by tissue-specific activation (Gaye *et al.*, 1986; Lesueur, 1990). Like other mammary tissue-specific promoters, activation of BLG promoter is also induced by lactogenic hormones (Kim *et al.*, 1995a; Gaye *et al.*, 1986; Lesueur, 1990). A previous study on the hormonal regulation of the caprine BLG promoter suggests that prolactin and glucocorticoid induce the BLG promoter directly while insulin may work indirectly (Kim *et al.*, 1995a).

Specific transcriptional activation, however, may not be the only mechanism that determines tissue-specificity. Repression seems to insure restricted expression of the BLG in mammary cells. In transgenic mice, the ovine BLG gene was expressed ectopically in salivary glands as the 5′-regulatory sequence was reduced down to -406 (Burdon *et al.*, 1994). Moreover, the expression of the transgene with 146 promoter was further diffused to such non-mammary organs as liver, kidney and salivary gland (Whitelaw *et al.*, 1992). These results indicate that repression by the upstream flanking sequence is as much important

as the specific activation in regulation of the BLG promoter in mammary tissue. Without repression, the BLG gene will be activated ubiquitously by the cell-type independent proximal activation. To guarantee temporal specificity in BLG expression, the BLG promoter should be silenced even in mammary cell till the cells become differentiated overtly. In the present study, the repression activity of the upstream regulatory sequence of the caprine BLG gene was examined in a transient expression in mammary HC11 cells, in which any activation derived from differentiation had not been reported previously (Doppler *et al.*, 1989). Moreover, the upstream sequence from −470 to −205 showing strong repression was investigated through electrophoretic mobility shift assay (EMSA) and DNase I protection assay to determine the negative regulatory element (NRE) within the sequence.

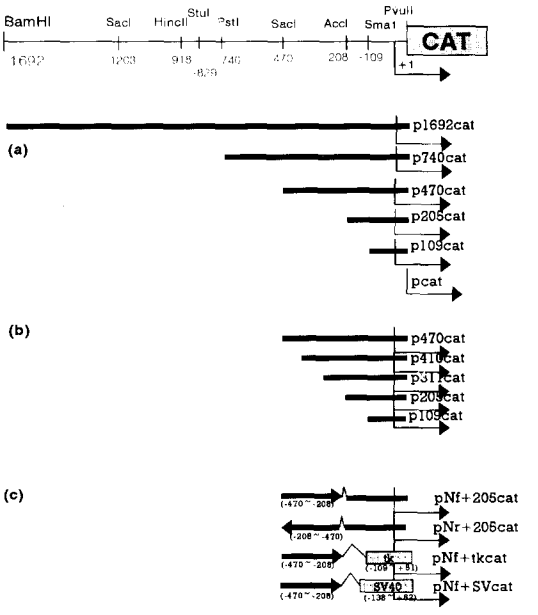

Fig. 1. Constructions of the expression vectors. (a) A series of expression vectors which was constructed by sequential truncation at appropriate restriction sites on the 1700bp 5′-regulatory sequence of the caprine BLG promoter. (b) The set of expression vectors used in Figure 3 was made by progressive digestion of the 5′-regulatory sequence of the p470cat vector. (c) Construction of the expression vectors with the regulatory sequence from −470 to −208 of the caprine BLG promoter in forward or reversed orientation. The direction of the thick arrow represents orientation.

Materials and Methods

Construction of expression vectors

The 5′-regulatory sequence from −1692 to +32 of the caprine BLG gene (Kim *et al.*, 1995b) was fused to the chloramphenicol acetyltransferase (CAT) gene. The recombinant gene was inserted to the pBluescript KS(+) (Stratagene) to construct the p1692cat expression vector. A series of deletion vectors were made by deleting progressively the distal end of regulatory sequence from the p1692cat vector (Fig. 1a). The expression vectors, p740cat, p470cat, p410cat, p109cat and pcat were constructed by truncating 5′-regulatory sequence at −740 PstI site, −470 *Sac*I site, −410 *Pst*I site, −109 *Sma*I site and +32 *Pvu*II site, respectively. The vectors p311cat, p205cat, p123cat were constructed by directional removal of nucleotides from 5′ end of the regulatory sequence of p470cat vector with *Exo*III-Mung bean kit (Stratagene). To confirm repression activity on the region from −470 to −205, the *Sac*I-*Acc*I fragment was fused to p205cat in forward and reversed orientation to construct pNf+205cat and pNr+205cat vectors. The same fragment was ligated to the 5′-end of the promoters in ptkcat and pSVcat vectors to construct such expression vectors, pNf+tkcat, pNr+tkcat, pNf+SVcat and pNr+SVcat. The ptkcat vector contains herpes simplex *thimidine kinase (tk)* promoter −109 to +51; McKnight, 1980) fused to chloramphenicol acetyltransferase (CAT) gene. The pSVcat was derived from pSV2cat and contains SV40 early promoter from −138 to +66 (Gorman *et al.*, 1982).

Cell culture

The CV-1 cells and HeLa cells were cultured in D-MEM (Gibco) media supplemented with 10% fetal bovine serum (Gibco). The HC11 cells (Ball *et al.*, 1988) were grown in growth media, RPMI 1640 (Gibco) media containing 10% fetal bovine serum and 10 ng/ml mouse epidermal growth factor (Sigma). Culture media was changed every

two days. For transient expression assay, the cells were cotransfected with 20 μg of the expression vectors and 2 μg of pCH110 (Pharmacia), β-galactosidase expression vector, by calcium phosphate method (Sambrook *et al.*, 1989). About 3 to 7 h 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 media and incubated for 16 h. The cells were shocked with 15% glycerol media and were cultured in growth media for 48 h and harvested to assay CAT activity (Sambrook *et al.*, 1989). The activity of β-galactosidase was determined with 1/2 or 1/4 of the cell extracts. The extract was added to the reaction mixture (67 mM Na phosphate, 1 mM $MgCl_2$, 45 mM 2-mercaptoethanol, 0.26 μg ONPG) and incubated until O.D.420 reach above 0.1 . The CAT activity was normalized to the β-galactosidase activity (Sambrook *et al.*, 1989).

Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared according to the procedure described in Current Protocols in Molecular Biology (Ausubell *et al.*, 1987b). All procedure were performed in cold room at 4°C. The harvested cells were allowed to swell in hypotonic buffer on ice and disrupted with Dounce homogenizer by striking 30 to 50 times with B pestle. After checking the cell disruption of about 90% with Trypan Blue dye, the nuclei were pelleted and extracted with high salt buffer containing 1.2 M KCl. Final concentration of KCl was 300 mM. The extract was dialyzed in dialysis buffer for 1 hour and the precipitate was removed by centrifugation. The supernant of the extract was aliquoted and stored at −70°C freezer before use after being frozen in liquid nitrogen. Quantitation of total protein of the extract was made by Bradford method (Ausubell *et al.*, 1987c). The probe was labeled with ^{32}P by filling its recessed 3′-ends with klenow fragment. For EMSA, the nuclear extract of 2 μg of total protein was diluted with dialysis buffer to 6 μl and mixed with 1 μl of 2 μg poly (dI:dC) and 1 μl of 3 mg BSA on ice. The mixture was let stand at room temperature for five minutes and then the probe of 20,000 cpm in 2 μl was added. For competition assay, about 100-fold in molar ratio of competitors were mixed just before adding the probes. The volume of extract was increased to 9 μl with dialysis buffer and final volume to 15 μl to adjust volume ratio. The binding mixture was incubated for 20 minutes at room temperature. The mixture was separated on non-denature gel of 4% polyacrylamide at 4°C After electrophoresis, the gel was dried and autoradiography was performed.

Foot printing

The radio-labeled probe was prepared by filling only one side with ^{32}P-labeled nucleotide. The labeled probe was isolated from the free nucleotides by Elu-tip (S and S Co, Germany) elution. 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), 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 minutes at room temperature (25°C). Then 50 μl of DNase I reaction buffer containing 10 mM Ca^{++} and 5 mM Mg^{++} was added. The mixture was digested by adding 2 μl of DNase I (Boeringer-Manheim, Germany) 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, 5M NaCl, 0.5M EDTA and tRNA). The resulting samples were extracted with phenol-chloroform (1:1) mixture and precipitated with ethanol. The precipitant was dissolved in a loading buffer (95% formamide, etc.). As the sequence references, C+T and C reaction of Maxam-Gilbert sequencing was performed according to the supplier's manual (Sigma Co., USA). The DNase I reaction samples were separated on 6% urea-polyacrylamide gel.

Results

Negative regulation of the caprine BLG promoter

In order to identify the regions that participate in regulation of the caprine BLG gene expression

in mammary cells, deletion analysis of the 5′-regulatory sequence of the gene was performed in transient expression in HC11 cells. The proximal regulatory sequence from -205 to -109 activated the downstream caprine BLG promoter strongly in transient expression in HC11 cells (Fig. 2). Such activation was suppressed in both growing and confluent cells by the adjacent upstream flanking sequence from -205 to -470. The sequence reduced the CAT expression level to about 30% of the maximum expression of p205cat. In growing HC11 cells, the CAT expression was further reduced to 10% of the maximum by the sequence upstream of -740. In confluent cells, however, the distal upstream sequence relieved the downstream repression activity slightly.

To confirm the repression activity of the sequence from -470 to -205, the sequence was translocated or reversed in orientation on the upstream of 205 promoter. When the sequence was dislocated by inserting 24 bp multicloning site, the sequence still retained repression activity in both non-mammary CV-1 and mammary HC11 cells although the degree of repression was decreased (pNf+205cat in Fig. 3a). The repression activity of sequence persisted even in reversed orientation (pNr+205cat in Fig. 3a). In all cases the repression in HC11 cells was not as

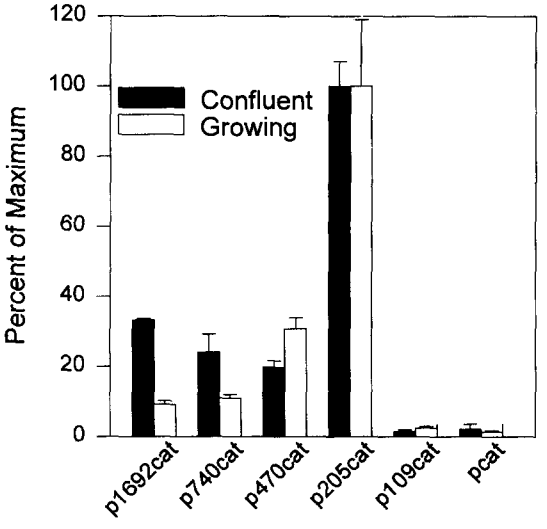

Fig. 2. Transient expression of a series of 5′-deletion vectors in mammary HC11 cells. A series of 5′-deletion vectors (constructions in Figure 1a) was transfected to HC11 cells and expressed transiently. The promoter activity was determined by measuring the CAT expression. The experiment was repeated several times and data shown are average of duplicates.

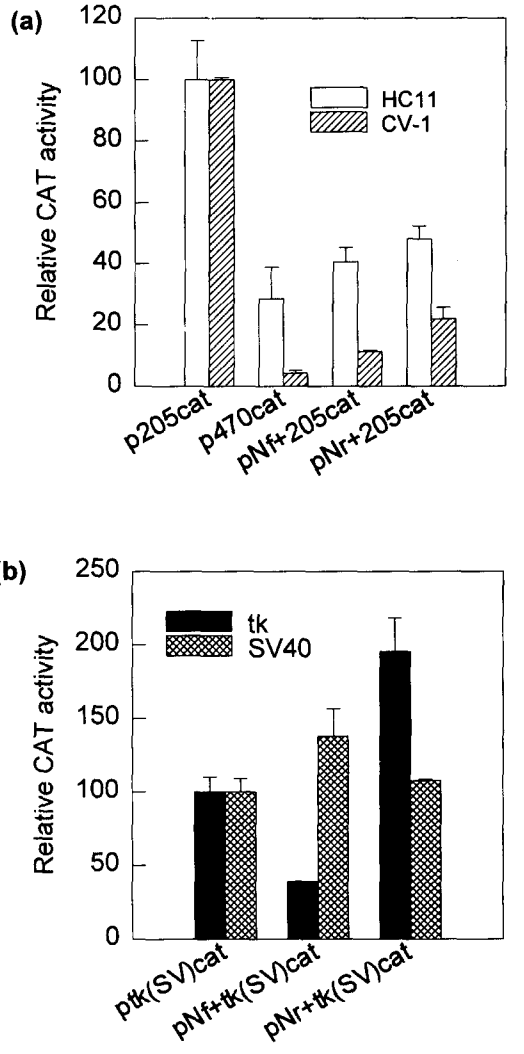

Fig. 3. Confirmation of repression activity on the sequence from -470 to -208. (a) The recombinant genes with caprine BLG 205 promoter (constructions in Figure 1c) were expressed transiently in mammary HC11 cells or non-mammary CV-1 cells. (b) The vectors with *tk* or SV40 promoter were expressed in CV-1 cells transiently. Data shown are representatives of several repeated experiments.

intensive as in CV-1 cells. The effect of the putative negative regulatory element of the caprine BLG on heterologous promoters, however, varied. The sequence could repress the heterologous *tk* promoter activity in CV-1 cells when located in forward orientation (Fig. 3b). Its repression activity, however, was switched to activation in reversed orientation. Similar repression and activation by the upstream regulatory sequence depending on the orientation was also observed in HeLa cells (data not shown). The sequence activated SV40 early promoter in forward orientation.

Localization of the negative regulatory elements (NREs)

For the purpose of localizing the essential region that represses the downstream caprine BLG promoter, the sequence from -470 to -109 was deleted progressively from 5′-end (Fig. 4). In HeLa cells, the proximal activation by the 205 promoter was suppressed to 20% level by the sequence from -311 to -205. A similar level of suppression by the sequence from -311 to -205 was also observed in CV-1 cells. In CV-1 cells, however, a further decrease in promoter activity by the sequence from -470 to -410 was observed.

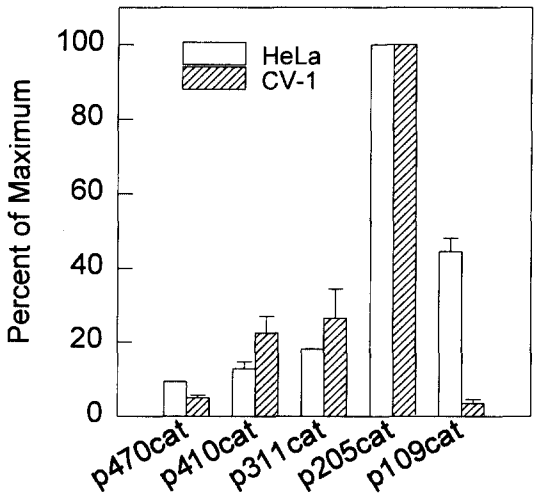

Fig. 4. Localization of the negative regulatory elements (NREs). The HeLa and CV-1 cells were transfected with expression vectors indicated (constructions in Figure 1b) and cultured for 48 hours before harvest. Data shown are average of two independent experiments that were duplicated.

To confirm that repression by the upstream regulatory sequence was accomplished by binding of trans-acting factor, the sequence was divided into two fragments, from -470 to -365 and from -365 to -208, and subjected to electrophoretic mobility shift assay (EMSA). Both fragments were shifted strongly with the nuclear extracts from the non-mammary HeLa and CV-1 cells, but not with that of mammary HC11 cells (Fig. 5). Moreover, both fragments appeared to bind factors of similar molecular mass. The nuclear extract from HeLa cells formed two complexes with both regulatory fragments. The CV-1 extract formed only one complex with the sequence from -470 to -365. Its mobility was coincided to the fast-moving complex in HeLa extract (Fig. 5a). The sequence from -365 to -208, however, formed two complexes of similar mobilities when complexed with the extract from CV-1 cells. Their mobilities were slightly faster and slower than the fast-moving one in HeLa cells (Fig. 5b). The extract from the mammary HC11 cells, on the other hand, retarded the mobility of the regulatory sequences only weakly. The mobilities of complexes formed by the HC11 extract were agreed to those of CV-1 extract.

To identify the regulatory elements that were recognized by the binding factors, the sequence from -365 to -208 was protected with HeLa and CV-1 extracts and subjected to DNase I digestion (Fig. 6). In spite that the two extracts formed different complexes of distinct mobilities, no perceptible differences was observed in protected regions. In the analysis, at least four regions, from -225 to -232, from -243 to -267, from -272 to -288 and from -301 to -316, were protected from DNase I digestion. Among the four protected regions, the region from -272 to -288 was protected most preferentially. The adjacent sequence from -290 to -300 became slightly more prone to DNase I digestion. The common or homologous sequence between the protected regions was not recognized.

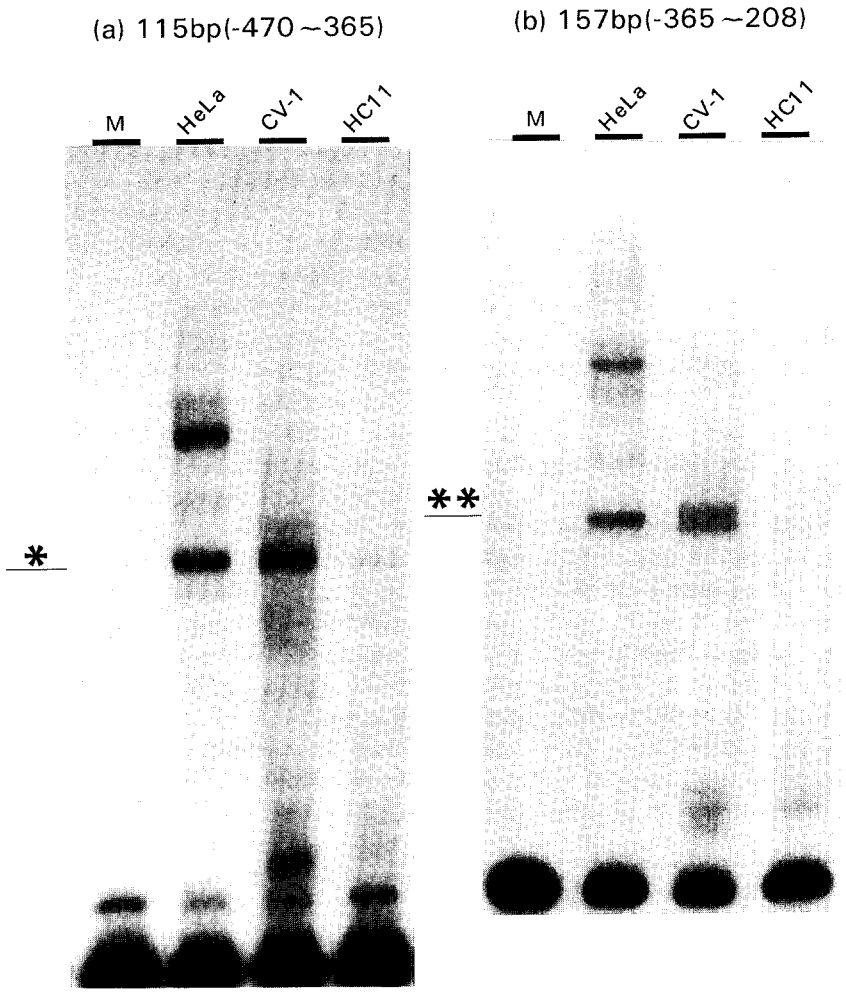

Fig. 5. Electrophoretic mobility shift assay (EMSA) of the putative negative regulatory elements (NREs). The fragments from -470 to -365 (a) and from -365 to -208 (b), both of which involve the regions having repression activity, were mixed with the nuclear extract prepared from the cells indicated. M indicate the lane for mock binding mixture. The stars indicate the fast-moving complexes having similar mobilities in non-mammary HeLa and CV-1 extracts.

Discussion

Expression of BLG in mammary tissue exhibits temporal and spatial specificity. Repression seems to participate in determining not only the spatial or tissue-specificity but the temporal or developmental stage-specificity. Repression of the caprine BLG promoter by the sequence from -470 to -205 was observed in non-mammary HeLa and CV-1 cells (Fig. 3a) as well as in mammary HC11 cells in transient expression (Fig. 2). In transient expression, even the cells kept confluent for two days revealed obvious repression. Because the cells were maintained in actively dividing status in transient expression, the cells in the system seems to be closer to the undifferentiated status than the cells in stable expression. Derepression observed in stable expression may require longer maintenance in full confluence. Alternatively, chromosomal integration that is attained in stable expression may be required for derepression in HC11 cells.

Repression of BLG promoter activity mediated by the upstream flanking sequence was strongly

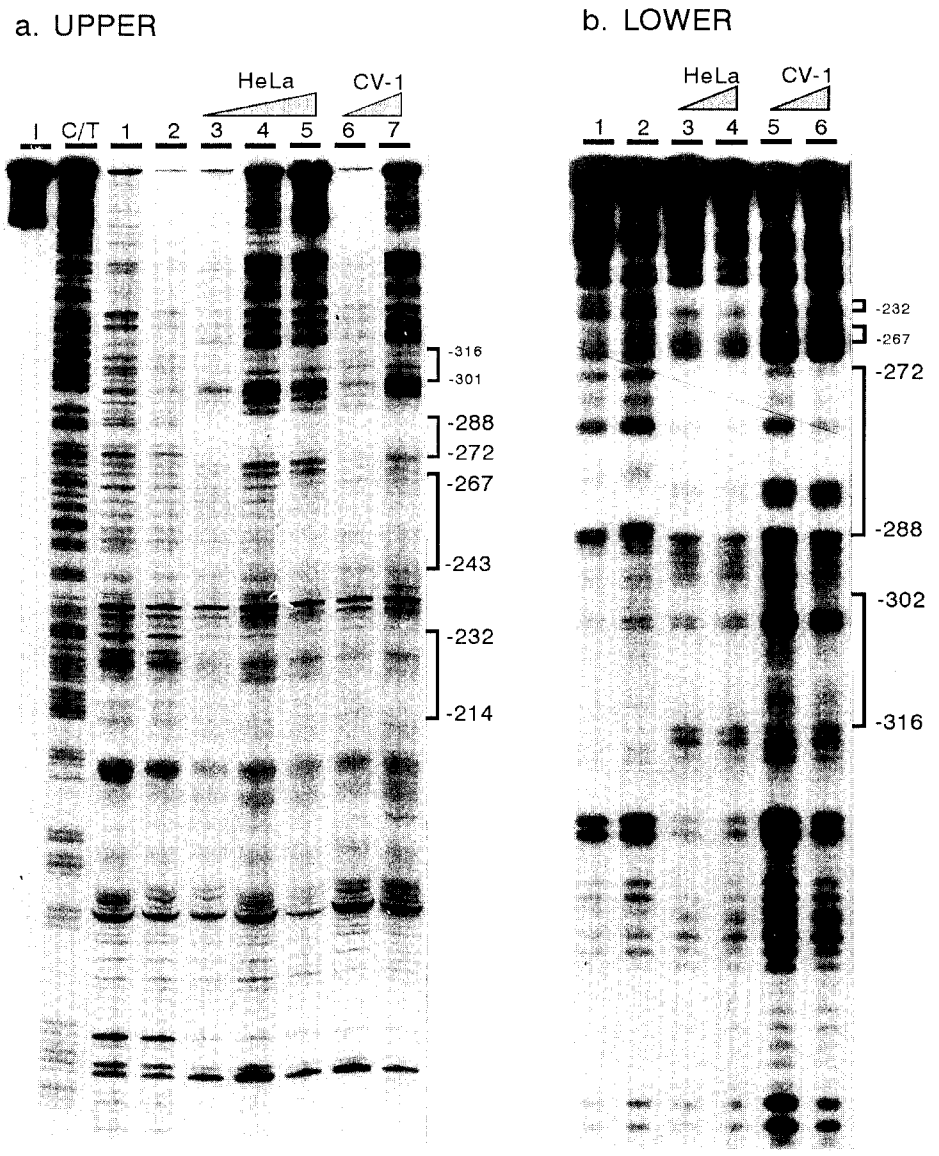

Fig. 6. Comparison of foot printing of the regulatory sequence from −109 to −470 with the extract of the HeLa and CV-1. Both the coding (a. upper) and non-coding (b. lower) sequences are shown. Preparation of the probes, sequencing and negative control reactions were carried out as described in *Materials and Methods*. The probes were protected with 40 μg lane 3a), 80 μg lane 4a, 6a, 3b, 5b) and 120 μg (lane 5a, 7a, 4b, 6b) of nuclear extracts from the cells indicated and digested with 2U of DNase I. The protected regions within the region from −208 to −365 are indicated with the numbers of the bases at both ends. The letter, I indicates intact lane.

correlated to the binding of the nuclear factors on the sequence. The sequence from −470 to −208, which exhibited repression, were bound by factors in non-mammary cells. The binding intensity was in parallel with the degree of repression. Such a correlation suggests that the binding factor may downregulate the activity of the downstream BLG promoter. The results, however, reject the possibility of direct repression by competing for binding sites with a transcriptional activator in

regulation of BLG promoter activity (Clark and Docherty, 1993).

Although the upstream regulatory sequence repressed downstream BLG promoter in either orientation (Fig. 3a), the sequence did not always repress downstream heterologous promoter. When the sequence was fused to *tk* promoter, it repressed the downstream promoter in forward orientation but activated in reversed orientation (Fig. 3b). The same sequence even activated downstream heterologous SV40 early promoter. Some structural difference among various promoters may explain the observed difference in regulation. Both the BLG and *tk* promoter have two GC-rich regions around −110 and −60 (Kim *et al.*, 1995b; McKnight, 1980). In SV40 promoter, however, three GC-rich regions occur in a tendom repeat on the region from −100 to −50 (Davidson *et al.*, 1986). The results suggest that the repression may be accomplished not by the binding itself. The context of the binding factors on the regulatory sequence appears to determine whether the binding results in transcriptional repression or activation.

The binding factors in non-mammary HeLa and CV-1 cells formed complexes of distinguished mobilities. The binding factors, however, did not reveal any prominent differences in DNase I protection assay. The result implies that the repression in HeLa and CV-1 cells might be mediated by the same element or the same binding factor. The recognized sequence, however, does not involve any known negative regulatory element such as YY1, Yin and Yang 1 (Kim *et al.*, 1995b; Raught *et al.*, 1994; Meier and Groner, 1994). Further investigation on transcriptional repression of the BLG promoter will help characterize the negative regulatory elements that secure tissue-specific repression.

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염소 베타-락토글로불린 유전자 프로모터의 음성 조절 인자 규명

김재만[1,2] · 유명희[1](1한국과학기술연구원 생명공학연구소;
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염소 베타-락토글로불린 유전자 프로모터의 유선 조직 특이성은, 비 발현 세포에서 −470 에서 −205 부위에 의해서 매개되는 억제 조절에 의해서 보증된다. 이 음성 조절 기작과 억제 조절을 매개하는 인자를 확인하기 위하여 상류 염기 서열을 자세히 분석하였다. 상류 염기 서열은 어느 방향으로 위치하든 연결된 베타-락토글로불린 유전자 프로모터의 활성을 억제할 수 있었다. 이와 같이 규명된 염소 베타-락토글로불린 유전자의 잠정적 음성 조절 인자는 다른 유전자의 프로모터들에 대해서는 다양한 활성을 보였는데, herpes simplex 바이러스의 *thimidine kinase* 프로모터는 상류 염기서열의 방향에 따라 억제 또는 활성화되었으며, SV40 프로모터는 억제되기보다는 오히려 활성화되었다. 염소 베타-락토글로불린 유전자의 억제·조절 인자를 포함하는 조절 부위는 비 유선 세포인 HeLa 및 CV-1 세포에서 추출된 핵 추출물에 의해서 이동성이 강력하게 지연되는 반면, 유선세포인 HC11 세포의 핵 추출물에 의해서는 약하게 지연되었다. 이와 같은 활성 억제와 인자 결합간의 연관성은 foot-printing 분석에서 관찰된 결합 부위가 염소의 베타-락토글로불린 유전자의 조직특이적 억제에 작용할 음성 조절 인자일 가능성을 시사한다.