Yin-Yang 1 Negatively Regulates the Differentiation–Specific Transcription of Mouse Loricrin Gene in Undifferentiated Keratinocytes.

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Abbreviations: YY1, Yin-Yang 1 ; AP-1, activator protein-1; Sp1, stimulator protein 1, EMSA , Electrophoretic Mobility Shift Assay;

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Abstract

Loricrin is a major component of the epidermal cornified cell envelope, and is expressed only in terminally differentiated keratinocytes. This cell differentiation-specific expression pattern suggests specific suppression of loricrin gene expression in undifferentiated keratinocytes as well as its activation in differentiated keratinocytes. We identified a negative regulatory sequence element in the first intron of the mouse loricrin gene involved in suppression of loricrin gene expression in undifferentiated keratinocytes. A database search indicated that this sequence contained the putative inverted YY1 binding motif. Constructs with point mutations in the putative YY1 binding motif showed increased reporter activity, indicating that YY1 negatively regulates loricrin gene transcription. Co-transfection experiments using a YY1 expression vector revealed that YY1 represses loricrin promoter activity. Western blotting and immunohistochemical analyses indicated that YY1 is more abundant in undifferentiated than in differentiated keratinocytes. These findings suggest that YY1 contributes to specific loricrin gene expression in differentiated keratinocytes by suppression of its transcription in undifferentiated keratinocytes. Furthermore, we demonstrated that forced expression of YY1 in differentiated keratinocytes results in specific down-regulation of expression of other early and late differentiation markers.

Introduction

The mammalian epidermis is a constantly renewing stratified squamous epithelium. Epidermal keratinocytes are the major cell type in the epidermis and are responsible for generating the protective barrier as a consequence of a tightly regulated, highly compartmentalized differentiation process that culminates in a mature stratified epidermis (Roop, 1995). Differentiation proceeds from the basal layer, through the spinous layer, granular layer, and ultimately the stratum corneum where fully differentiated keratinocytes are sloughed off into the environment as flattened squames. As keratinocytes differentiate into granular-layer cells, they show induction of late-differentiation products, such as filaggrin (Fisher *et al*, 1987; Rothnagel *et al*, 1987), involcrin (Rice and Green, 1979), and loricrin (Mehrel *et al*, 1990). This latter molecule, loricrin, is a major component of the cornified cell envelope, a specialized structure that replaces the plasma membrane and contributes to the protective barrier function of the stratum corneum (Koch *et al*, 2000). Loricrin is expressed in a keratinocyte- and differentiation-specific manner (Steven and Steinert, 1994), and *in situ* hybridization analyses have shown that loricrin transcripts are localized only in the upper spinous and granular layers of the epidermis (Mehrel *et al*, 1990; Hohl *et al*, 1991b). Loricrin gene expression is strictly regulated mainly at the level of transcription. Expression of loricrin has been shown to be induced on induction of differentiation of cultured keratinocytes by increasing the calcium concentration in the culture media (Hohl *et al*, 1991a). Elevation of calcium concentration in the culture medium triggers differentiation, as reflected by the expression of differentiation markers, such as keratins 1 and 10, loricrin, and involucrin. The calcium switch model thus allows simulation of *in vivo* differentiation. Using this inducible culture system, the tissue- and differentiation-specific expression of loricrin provides an ideal experimental model in which to study the transcription factors involved in the control of keratinocyte- and differentiation-specific gene expression. It has been reported that 6.5 kb of upstream sequence is required for epidermis-specific expression of the mouse loricrin gene, and an evolutionarily highly conserved

AP-1 element in the loricrin proximal promoter is essential but not sufficient for loricrin expression (DiSepio *et al*, 1995). Further experiments demonstrated that the Sp-1 binding site located immediately upstream of the AP1 site in the loricrin proximal promoter is also essential for loricrin expression, and molecular interactions of SP-1/Sp-3 and AP1 proteins suggest a simple model for regulation of the loricrin proximal promoter (Kawachi *et al.* unpublished data). However, the precise mechanism of regulation of specific loricrin gene expression is still unclear. Little is known about the molecular mechanism involved in suppression of loricrin expression in basal epidermal keratinocytes.

In the present study, we found that YY-1, a constitutive nuclear member of the GLI-Krüppel family of zinc finger transcription factors, is expressed dominantly in undifferentiated keratinocytes, and contributes to specific suppression of loricrin gene expression in undifferentiated keratinocytes. Furthermore, we report here that YY1 also suppresses the expression of other differentiation marker proteins, such as keratin 1, keratin 10, and involucrin in differentiated keratinocytes.

Materials and Methods

Plasmids The isolation and characterization of a 14-kb genomic loricrin gene fragment has been reported elsewhere (DiSepio *et al*, 1999). A 2.6-kb loricrin genomic fragment containing 1.5 kb of upstream regulatory sequence, the TATA box, 47 bp of the untranslated exon 1, and 1.1 kb of intron 1 was inserted into the plasmid pGL3-basic (Promega, Madison, WI) in-frame with the promoter-less luciferase reporter cassette (firefly luciferase) to generate 1.5-1.1LOR. To obtain a series of intron 1 deletion constructs, 1.5-1.1 LOR was subjected to *Eco*RV/*Nsi*I digestion, which eliminated a 371-bp fragment yielding the construct DEL1, and *Pst*I/*Eco*RV digestion, which eliminated a 374-bp fragment yielding the construct DEL2 (Fig. 1). To generate constructs DEL3, DEL4, and DEL5, the specific fragments between the *Pst*I and *Eco*RV sites in intron 1 were amplified by PCR using 5’ primers corresponding to positions 200, 250, or 300 bp upstream from

the *Eco*RV site each of which contained an artificial *Pst*I site. PCR was performed using the common 3' primer that included the *Eco*RV site. The PCR product was ligated with the DEL2 construct digested with *Pst*I and *Eco*RV (Fig.1). For point mutagenesis of the YY1 recognition site, the 5' primer with an artificial *Pst*I site and a point mutation on the YY-1 recognition sequence (Fig. 1 MUT) was generated. PCR and ligation reactions were performed using 5' *Pst*I I and 3' *Eco*RV sites as described above. The mammalian expression vector pCMV-YY1 was kindly provided by Dr. Yang Shi (Harvard Medical School, Boston, MA). All deletions and mutations were confirmed by DNA sequencing.

Cell culture and transient cell transfection Primary mouse keratinocyte cultures were prepared as described previously (Rothnagel *et al*, 1993). Primary keratinocytes were isolated and then grown in low-calcium medium (0.05 mM calcium, 50% fibroblast-conditioned medium (Harper *et al*, 1988; Rothnagel *et al*, 1993), 8% FCS in EMEM (BioWhittaker, Walkersville, MD)) for 48 hours prior to transfection. Then, keratinocytes were cultured in either high-calcium medium (0.12 mM calcium, 50% fibroblast-conditioned medium, 8% FCS in EMEM) or in low-calcium medium. In induction of terminal differentiation, keratinocytes were still more cultured in very high-calcium medium (0.35 mM calcium, 50% fibroblast-conditioned medium) for 24 hours after culture in high-calcium medium. Low-calcium medium was supplemented with 4.0 ng/ml of EGF (Becton Dickinson, Bedford, MA) unless otherwise indicated. Mouse dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected using the LT1 reagent in accordance with the manufacturer's recommendations (Panvera, Madison, WI). To examine expression in undifferentiated keratinocytes, cells were cultured in low-calcium medium for 48 hours after transfection and then harvested. Expression in differentiated keratinocytes was assessed by growing transfected cells for 24 hours in low-calcium medium without EGF, followed by two days in high-calcium medium. To determine transfection efficiencies and to normalize expression levels, all cultures were co-transfected with plasmid pRL-TK (Promega). This plasmid contains the *Renilla* luciferase gene

under the control of the HSV TK promoter. Luciferase assays were performed using a dual-luciferase assay kit according to the manufacturer's protocol (Promega). Firefly and sea pansy luciferase activities were measured with a Monolight 2020 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Each construct was tested in triplicate.

Electrophoretic Mobility Shift Assays (EMSA) Whole-cell extracts were obtained from dissociated epidermal keratinocytes. Cells were washed with PBS, resuspended in storage buffer (20 mM HEPES, pH 7.8, 400 mM KCl, 20% glycerol, 2 mM DTT), and then frozen in a dry ice/ethanol bath. Supernatants were thawed on ice, centrifuged at 15,000×g, and aliquoted. Nuclear extracts were prepared from differentiated or undifferentiated keratinocytes as described previously (Schreiber *et al*, 1989). Protein concentrations were determined with a Bradford assay kit (BioRad, Hercules, CA), and equal amounts of protein were used for each DNA/protein binding reaction. Oligonucleotides were end-labeled using ^{32}P-ATP and T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Labeled probes (2 ng; 10^5-10^6 cpm) were incubated with 1-3 μg of cell extract for 15 to 30 minutes on ice. In some experiments, 1 μg/reaction of anti-YY1 antibody (sc-1703X; Santa Cruz Biotechnology, Santa Cruz, CA) was added. The DNA/protein complexes were analyzed by native polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography of the dried gels. The sequence of radiolabeled oligonucleotide for YY1 binding corresponded to the region of +400 to +425 on the first intron of the loricrin gene. The sequence of the mutant YY1 binding oligonucleotide is shown in Fig. 1C as MUT. Oligonucleotides with the consensus binding sequence for cold competition experiments and anti-actin antibody (sc-7210) as a control antibody for supershift assay were purchased from Santa Cruz Biotechnology (sc-2533).

Immunohistofluorescence staining Immunohistofluorescence staining was performed using an antibody to YY1 (sc-1703, Santa Cruz Biotechnology). Skin specimens obtained from neonatal mice were embedded in Tissue-TekII OCT compound (Sakura Finetek, Torrance, CA) and frozen. Sections 5 μm thick were cut on a cryostat, and placed on uncoated slides. For staining, slides were

incubated with primary antibody for 30 min, followed by washing with PBS. Slides were incubated with fluorescein isothiocyanate-conjugated secondary antibody (sc-2012; Santa Cruz Biotechnology), mounted, and then analyzed by fluorescence microscopy (Nikon, Tokyo, Japan).

Immunoblotting analysis Whole-cell extracts were prepared from differentiated or undifferentiated keratinocytes. Nuclear extracts were prepared from differentiated or undifferentiated keratinocytes as described previously (Schreiber *et al*, 1989). For Western blotting analysis of the expression of pCMV-YY1, whole-cell extracts from the transfected keratinocytes were obtained 48 hours after transfection. Proteins were separated by 8.5%-10% SDS-PAGE and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blots were probed successively with antibodies to YY1 (sc-281; Santa Cruz Biotechnology), keratin 14 (PRB-155P; Covance, Richmond, CA), keratin 5 (PRB-160P; Covance), involucrin (PRB-142C; Covance), and loricrin (PRB-145P; Covance). After detection of primary antibody binding, blots were stripped using ImmunoPure IgG elution buffer (Pierce Chemical Company, Rockford, IL). Primary antibody binding was detected using PicoWest SuperSignal ECL Substrate (Pierce) and exposure to Biomax MR film (Kodak, Rochester, NY).

Results

Identification of the negative regulatory element in the first intron of the loricrin gene The expression of loricrin is strictly linked to keratinocyte differentiation, suggesting its specific repression in undifferentiated keratinocytes as well as activation in differentiated keratinocytes. To locate the negative regulatory sequences involved in suppression of loricrin gene expression in undifferentiated keratinocytes, a series of 5’ deletions were made and placed upstream of the luciferase reporter gene. The constructs were analyzed by transient transfection into primary murine dermal fibroblasts or primary murine keratinocytes cultured under either non-differentiation (0.05 mM Ca^{2+}) or differentiation-inducing (0.12 mM Ca^{2+}) culture conditions. The loricrin gene consists of two exons, the 5’ non-coding first exon and the second 3’ exon

containing the entire coding sequence, separated by one intron. On transfection into differentiated keratinocytes, the construct containing the promoter region, the untranslated first exon, and the first intron (-1.5+1.1LUC) showed a decrease in reporter activity of over 50% as compared to those containing only the promoter region (-0.3LUC, -0.5LUC and -1.5LUC) (Fig. 1A). This result suggested that the first intron contains a negative-acting *cis*-element.

Further deletion analysis indicated that the region from +398 to +428 is responsible for the suppressive activity of the first intron (Fig. 1B, DEL1-5). A transcription factor database search identified a putative inverted binding motif for YY1 in this sequence (Fig. 1B, DEL5). The point-mutated constructs with contiguous 2-bp substitutions at the putative YY1-binding motif showed increases of about 50% in reporter activity (Fig. 1C, MUT/DEL5), indicating that YY1 negatively regulates loricrin gene transcription in keratinocytes.

YY-1 physically binds to the functional negative element in the first intron of loricrin gene

Electrophoretic mobility shift assays were performed to determine whether YY1 actually binds to the functional negative element. A fragment of 25 bp derived from the negative element in the first intron was used as a probe. One distinct DNA/protein complex was detected using nuclear extracts prepared from epidermal keratinocytes (Fig. 2, lane 1). Preincubation with 100-fold molar excess of an unlabeled wild-type oligonucleotide inhibited complex formation (Fig. 2, lane 3). Preincubation with even a 100-fold molar excess of unlabeled mutant oligonucleotide with 2-bp contiguous substitutions within the negative element failed to inhibit complex formation (Fig. 2. lanes 4 and 5). Unlabeled oligonucleotides containing a typical YY1 consensus binding site completely inhibited complex formation (Fig. 2, lanes 6 and 7). To confirm more directly that YY1 protein binds to the negative element, we added a polyclonal antibody against YY1 to the protein extracts in these electrophoretic mobility shift assays. As shown in Fig. 2, lanes 8 and 9, anti-YY1 antibody inhibited protein/DNA complex formation and generated supershifted bands. These experiments clearly demonstrated that YY1 can bind to the functional negative element in the first intron of the loricrin gene. Another shifted band (indicated by asterisks in Fig. 2) was observed that

was competed with the wild-type but not the mutant oligonucleotide. Interestingly, the typical YY1 consensus oligonucleotide did not compete with this band. These findings suggest that a DNA-binding factor other than YY1 also binds to the sequence of the negative regulatory element.

YY1 expression is down-regulated during keratinocyte differentiation Immunohistochemical staining of the epidermis was performed to investigate epidermal YY1 expression *in vivo*. YY1 was strongly expressed mainly in the cytoplasm in cells of the basal epidermal layer, while its expression was weak in the suprabasal layers (Fig. 3A). To examine the changes in level of YY1 protein in keratinocytes during differentiation, Western blotting analysis was performed using nuclear extracts prepared from undifferentiated and differentiated keratinocytes. As shown in Fig. 3B, YY1 was more abundant in the nuclear extracts of cells grown under non-differentiation (0.05 mM Ca^{2+}) than differentiation-inducing conditions (0.12 mM Ca^{2+}). To confirm down-regulation of the DNA-binding activity of YY1 by keratinocyte differentiation, the nuclear extracts of cells grown under non-differentiation and differentiation-inducing conditions were analyzed by electrophoretic mobility shift assay. As shown in Fig. 3C, the intensity of the shifted band was much higher in the lanes containing nuclear extracts from cells grown under non-differentiation (0.05 mM Ca^{2+}) than differentiation-inducing conditions (0.12 mM Ca^{2+}). These observations indicated that the DNA-binding activity of YY1 was elevated in undifferentiated keratinocytes and down-regulated in differentiated keratinocytes. This differentiation-dependent down-regulation of YY1 expression suggested that YY1 contributes to the specific expression of loricrin gene in differentiated keratinocytes by suppression of its transcription in undifferentiated cells.

Overexpressed YY-1 functionally suppresses loricrin promoter activity To determine the specific functional effect of YY1 on loricrin gene transcriptional activity in keratinocytes, transient co-transfection experiments were performed in cultured keratinocytes. The loricrin reporter construct p0.5-1.1 Luc containing 0.5 kb of 5’-flanking region and 1.1 kb of the first intron, was transfected into keratinocytes grown under non-differentiation or differentiation-inducing

conditions in the presence of the YY1 expression vector pCMV-YY1 or control empty vector. As shown in Fig. 4, YY-1 decreased loricrin reporter activity to less than 3% of the control level in both undifferentiated and differentiated keratinocytes. Thus, YY1 was shown to act as a potent negative regulator of loricrin gene expression.

YY1 decreases expression of keratinocyte differentiation marker genes Next, we analyzed the effects of YY1 on expression of endogenous differentiation marker genes in keratinocytes grown in low- or high-calcium medium. Western blotting analysis was used to monitor expression of the basal markers keratin 5 and 14, the early differentiation markers keratin 1 and 10, and the terminal differentiation markers involucrin and loricrin. Differentiating keratinocytes in the presence of a high level of calcium (0.12 mM) and terminally differentiated keratinocytes in the presence of a very high calcium level (0.35 mM) showed marked decreases in the expression of the early and terminal differentiation markers after transfection with YY1 expression vector,, respectively. Interestingly, there were no differences in the expression of the basal keratinocyte markers keratin 5 or 14 by transfection of YY1. Therefore, forced expression of YY1 in differentiated keratinocytes resulted in down-regulation of early and terminal differentiation markers, indicating that the early and late differentiation markers are specifically suppressed in differentiated keratinocytes in a YY1-dependent manner.

Discussion

A number of regulatory elements and transcription factors have been shown to be involved specifically in the regulation of cell type-specific or differentiation-specific epidermal gene expression. Several ubiquitous transcription factors, such as AP-1, AP-2, Sp1/3, and NFκB, have been shown to be involved in the regulation of the epidermal gene expression (Eckert *et al*, 1997a; Eckert *et al*, 1997b). It is interesting that ubiquitously expressed transcription factors appear to control the specific expression of epidermal genes. The AP-1 site 6.5 kb upstream of the cap site is essential for epidermis-specific mouse loricrin gene expression (DiSepio *et al*, 1995). A recent study indicated that synergistic interactions among multiple transcription factors are important for keratinocyte-specific expression of the human loricrin gene (Jang and Steinert, 2002). However, the mechanisms and factors involved in the specific suppression of loricrin gene expression in basal undifferentiated keratinocytes remain to be elucidated.

To identify the negative elements involved in regulation of loricrin expression in undifferentiated keratinocytes, we investigated the functional role of the first intron. Our results identified an YY1-binding motif in the first intron as a negative regulatory element. In this study, we showed that YY1 is expressed at high levels in the epidermal basal layer and that it suppresses transcription of the loricrin gene in basal undifferentiated keratinocytes.

YY1 is a member of the GLI-Krüppel family of transcription factors and several lines of evidence suggest that YY1 is a multifunctional transcriptional regulator, involved in initiation, activation, or repression of transcription depending on both the promoter and cellular context. YY1 mRNA and protein have been identified in a number of different tissues and cell types, suggesting that it is expressed both constitutively and ubiquitously (Shi *et al*, 1997). However, YY1 expression level increases rapidly in quiescent NIH3T3 cells in response to serum and insulin-like growth factor-1 (Flanagan, 1995). YY1 DNA-binding activity has also been shown to be regulated during differentiation. For example, YY1 DNA binding activity decreases during differentiation of human teratocarcinoma cells (Liu *et al*, 1994) and in myoblast differentiation (Lee, T.C. *et al*,

1992), but increases during aging (Adrian *et al*, 1996). In addition, to differentiation-specific gene regulation, a recent study also indicated that YY1 contributes to the T cell-specific expression pattern of CD3delta (Ji *et al*, 2002). The results of the present study indicated that YY1 protein is expressed at high levels in undifferentiated basal keratinocytes and is down-regulated during differentiation both *in vivo* and *in vitro*. The level of YY1 DNA-binding activity also decreased markedly during differentiation, suggesting that YY1 may regulate undifferentiated keratinocyte-specific gene expression. As YY1 protein is degraded by the calcium-activated protease calpain II and the 26 S proteosome during myoblast differentiation (Walowitz *et al*, 1998), the calcium-dependent, post-translational proteolytic mechanism might be important for the specific inactivation of YY1 during keratinocyte differentiation. In the present study, we showed that YY1 bound to the negative regulatory element in intron 1 of the loricrin gene and functionally repressed loricrin gene transcriptional activity. Taken together, our findings indicate that YY1 contributes to the specific suppression of loricrin gene expression in undifferentiated keratinocytes. Furthermore, we demonstrated that the overexpression of YY1 in keratinocytes cultured under differentiation-inducing conditions resulted in marked decreases in the expression of not only loricrin but also of the other early and late differentiation marker genes, keratin 1, keratin 10, and involucrin, while the levels of expression of the undifferentiated marker genes keratin 5 and keratin 14 were relatively unaffected. These findings suggest that YY1 plays an important role in maintenance of the undifferentiated phenotype in keratinocytes. A functional YY1-binding site was identified 1.9 kb upstream of the human keratin 1 gene (Lee *et al.*, unpublished data) and our database search indicated that human and mouse keratin 10 genes have a possible YY1-binding site in their promoter regions. However, the promoter regions of keratin 5 and 14 genes contain no putative YY1-binding sites. YY1 has been implicated in the repression of genes that are intimately involved in various differentiation processes (Shrivastava and Calame, 1994). Repression of gene expression by YY1 may ensure correct temporal and spatial expression of differentiation-specific molecules (Raich *et al*, 1995). The relief of YY1 repression of the skeletal α-actin promoter is

thought to be important in myoblast differentiation (Lee, T.C. *et al*, 1992). The globin gene switch process during hematopoiesis has been suggested to involve YY1 (Orkin, 1995). The ability of ubiquitous YY1 to suppress differentiation-specific gene expression may be due to its ability to antagonize differentiation-specific transcriptional activators. In addition, activation of cell cycle-related genes, such as c-Myc, by YY1 may contribute to the repression of differentiation-specific gene expression (Riggs *et al*, 1993).

YY1 has been shown to regulate the gene transcription through physical and functional interactions with various transcription factors and co-factors. The specific actions of the ubiquitously expressed factor YY1 can be largely explained by its interactions with a diverse array of other sequence-specific factors and co-factors (Shi *et al*, 1997). A number of epidermal key regulatory proteins, such as Sp1, C/EBPβ, and CBP/p300, have been shown to interact with YY1 (Lee, J.S. *et al*, 1993; Bauknecht *et al*, 1996; Thomas and Seto, 1999), suggesting that these interactions are important in determining the particular function of YY1 at a given promoter. Regulation of human loricrin gene expression has been shown to require interactions among a number of ubiquitous transcription factors, the fine balance of the availability of these proteins, and recruitment of the co-activator p300/CBP (Jang and Steinert, 2002). Previously, we reported that both AP-1 and Sp1/Sp3 elements located 14-40 bp upstream from the TATA box are crucial for differentiation-specific murine loricrin gene expression and that they interact both functionally and physically with each other (unpublished data). Our findings suggested that AP-1 and Sp1/Sp3 proteins form a core transcription complex that can strongly activate transcription from the loricrin gene promoter in differentiated keratinocytes. Thus, the repression of loricrin transcription by YY1 in undifferentiated keratinocytes may be due to the coordinated interaction between YY1 and the core transcription complex composed of AP-1 and Sp1.

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Figure legends

Figure 1

Identification of negative elements in the first intron of the loricrin gene (A) Transient expression of the luciferase reporter gene under the control of the mouse loricrin 5'-upstream sequence. Plasmids containing serial deletions of the loricrin gene 5'-flanking region (-1.5Luc, -0.5Luc, and -0.3Luc) or containing 0.5 kb of 5'-flanking sequence and 1.1 kb of the first intron (-0.5+1.1Luc) linked to reporter vectors carrying the firefly luciferase gene (Luc) were transiently transfected into differentiated keratinocytes cultured in high-calcium medium (0.12 mM Ca^{2+}; black bars), undifferentiated keratinocytes cultured in low-calcium medium (0.05 mM Ca^{2+}; open bars), or normal dermal fibroblasts (dotted bars). The vector nomenclature is based on the length of the 5' loricrin sequence in each construct (*e.g.*, –1.5Luc contained sequences from positions +1 bp to –1.5 kb). Cells were harvested and assayed for luciferase activity as described in Materials and Methods. Error bars represent one standard deviation of triplicate transfections. Differences in transfection efficiency were normalized relative to sea pansy luciferase activity produced by the co-transfected pRL-tk vector. The relative luciferase activities shown are the ratios of firefly luciferase activities derived from pGL3-loricrin promoter constructs to sea pansy luciferase activities from the pRL-tk vector. (B) Identification of an element that is critical for the negative activity of the first intron. Plasmids containing various deletions in the first intron of the loricrin gene (DEL1-5) or the wild-type first intron (WT) ligated into reporter cassettes carrying the firefly luciferase gene (Luc) were transiently transfected into differentiated keratinocytes. The arrow at DEL5 indicates the critical region (398-428) for the negative activity of the first intron. Relative luciferase activity was as defined in Fig. 1A. (C) Effects of the YY1-binding motif on negative transcriptional activity of the first intron. Differentiating keratinocytes were transfected with DEL5 or mutated reporter plasmid with contiguous 2-bp point mutation in the YY1-motif (MUT/DEL5). Mutated sequences in YY1-binding motifs (TG to CA) are shown in closed boxes.

Figure 2

YY1 binds specifically to the putative negative motifs in intron 1 of the loricrin gene Electrophoretic mobility shift assays were performed using radiolabeled wild-type oligonucleotide containing the putative YY1-binding motif and nuclear extracts from epidermal keratinocytes. Incubations were carried out in the presence or absence of a 10-fold or a 100-fold excess of unlabeled wild-type oligonucleotide (lanes 2 and 3), mutant oligonucleotide (lanes 4 and 5), or typical YY1-binding consensus oligonucleotide (lanes 6 and 7). Nuclear extracts were preincubated with 1 μg (lane 8) or 2 μg (lane 9) of anti-YY1 antibody followed by incubation with radiolabeled wild-type probe. The arrowhead indicates a potential supershifted band. Preincubation with anti-actin antibody was also performed to confirm the specificity of the anti-YY antibody (right panel). The protein-DNA complexes were separated by PAGE. The specific band corresponding to YY1-DNA complex is indicated by an arrow. The faster moving band, which showed partial competition with the wild-type and YY1 consensus oligonucleotides but not the mutant oligonucleotide, is indicated by an asterisk.

Figure 3

Expression profile of YY1 protein in epidermis and cultured keratinocytes. (A) Expression of YY1 in stratified epithelium *in vivo*. Frozen sections of murine neonatal back skin were immunostained with an antibody to YY1. The dotted line represents the basement membrane zone. CL, cornified layer; SL, squamous layer; BL, basal layer; DE, dermis. (B) Changes in expression of YY1 through differentiation of cultured keratinocytes. Nuclear extracts from cultured undifferentiated (0.05 mM Ca^{2+}) and differentiated (0.12 mM Ca^{2+}) keratinocytes were examined for YY1 protein expression by Western blotting analysis. Equivalent quantities of extract were subjected to PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with anti-YY1 antibody (upper panel). As an internal reference for relative amounts of protein, the blots were re-probed using anti-lamin A/C antibody (lower panel). (C) Changes in DNA-binding

activity of YY1 through differentiation of cultured keratinocytes. Electrophoretic mobility shift assays were performed using nuclear extracts from cultured undifferentiated (0.05 mM Ca^{2+}, lanes 1 and 2) and differentiated (0.12 mM Ca^{2+}, lanes 3 and 4) keratinocytes. Nuclear extracts from cultured undifferentiated (lanes 5 and 6) and differentiated (lanes 7 and 8) keratinocytes were preincubated with 1 μg of anti-YY1 antibody followed by incubation with radiolabeled wild-type probe.

Figure 4

YY1 suppresses loricrin promoter activity in transiently transfected keratinocytes. The loricrin reporter construct p0.5-1.1 Luc, which contains 0.5 kb of 5'-flanking region and 1.1 kb of intron 1, was transfected into non-differentiating or differentiating keratinocytes in the presence of YY1 expression vector, pCMV-YY1, or control empty vector. Open bars indicate the luciferase activity of the corresponding constructs in undifferentiated keratinocytes grown in low calcium medium (0.05 mM Ca^{2+}). Black bars indicate luciferase activity in differentiated keratinocytes grown in high-calcium medium (0.12 mM Ca^{2+}).

Figure 5

Effects of YY1 overexpression on the expression of keratinocyte differentiation markers. Total cell extracts from undifferentiated keratinocytes or differentiated keratinocytes transfected with YY1 expression vector or vehicle vector were analyzed by immunoblotting with specific antibodies for the indicated proteins: A, basal keratinocyte markers, keratins 5 and 14; B, early differentiation markers, keratins 1 and 10; C, terminal differentiation markers, involucrin and loricrin. Undifferentiated keratinocytes were grown in low-calcium medium (0.05 mM Ca^{2+}), and differentiated keratinocytes were grown in high- or very high-calcium media (0.12 mM/0.35 mM Ca^{2+}, respectively). NT, non-transfected keratinocytes; YY1, transfected with YY1 expression vector pCMV-YY1; Vehicle, transfected with empty vehicle vector, pCMV.

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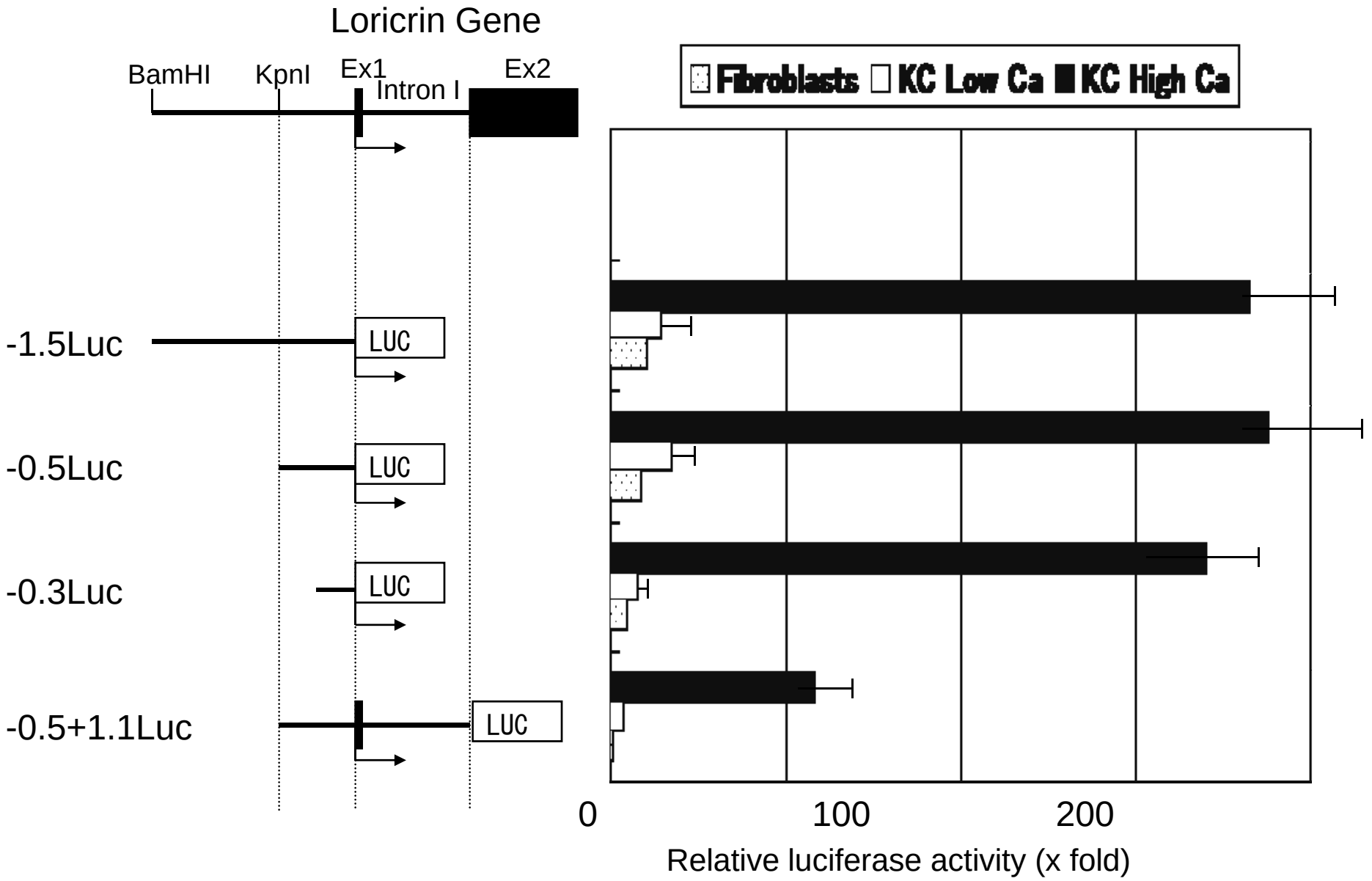
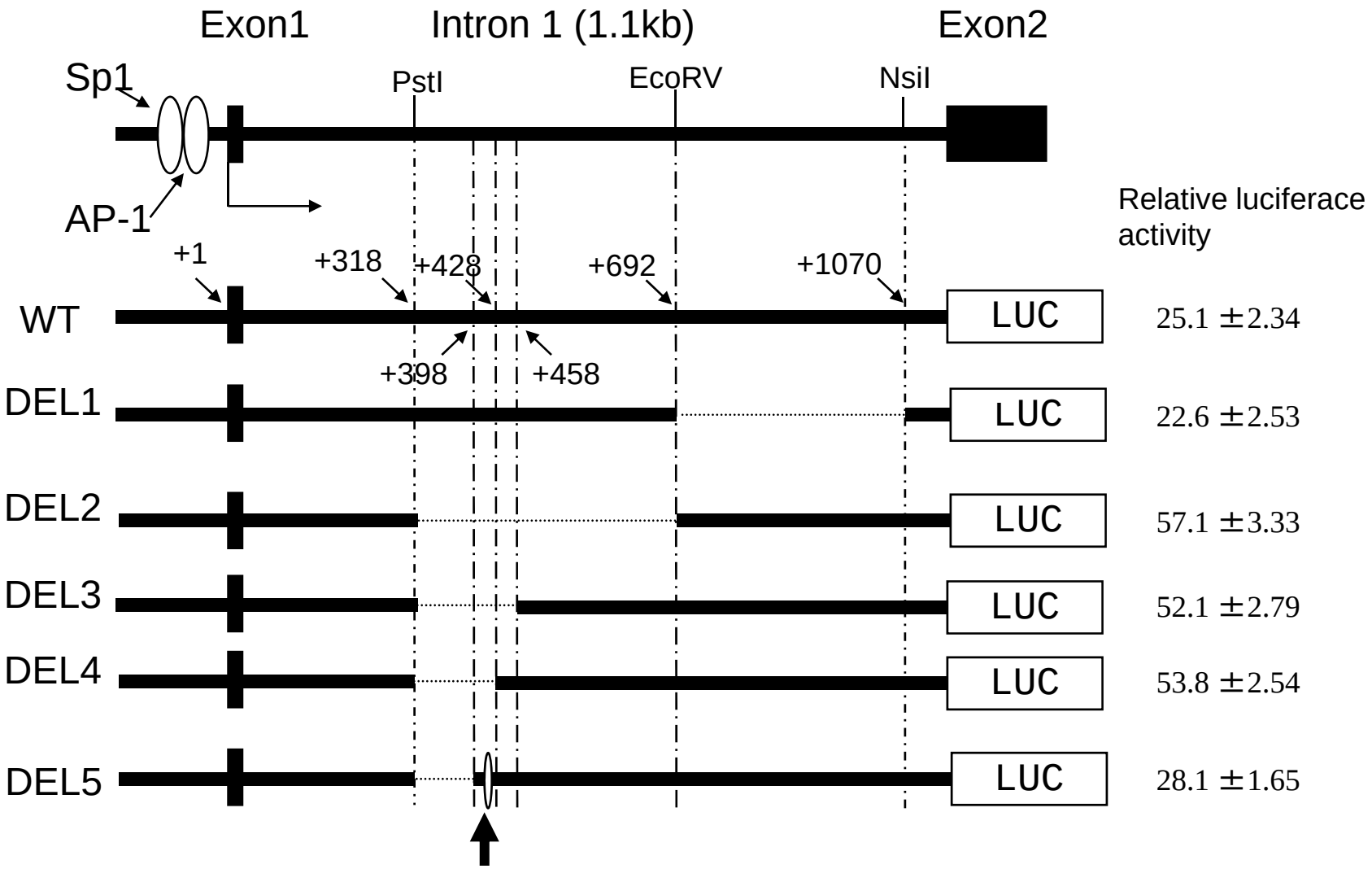
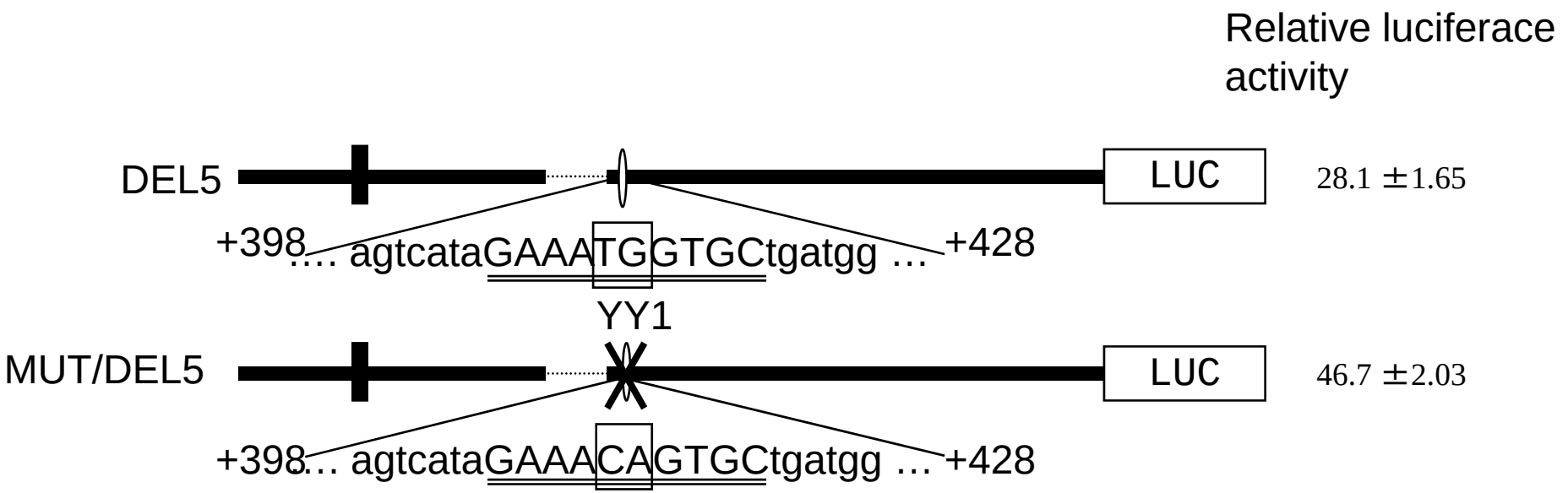
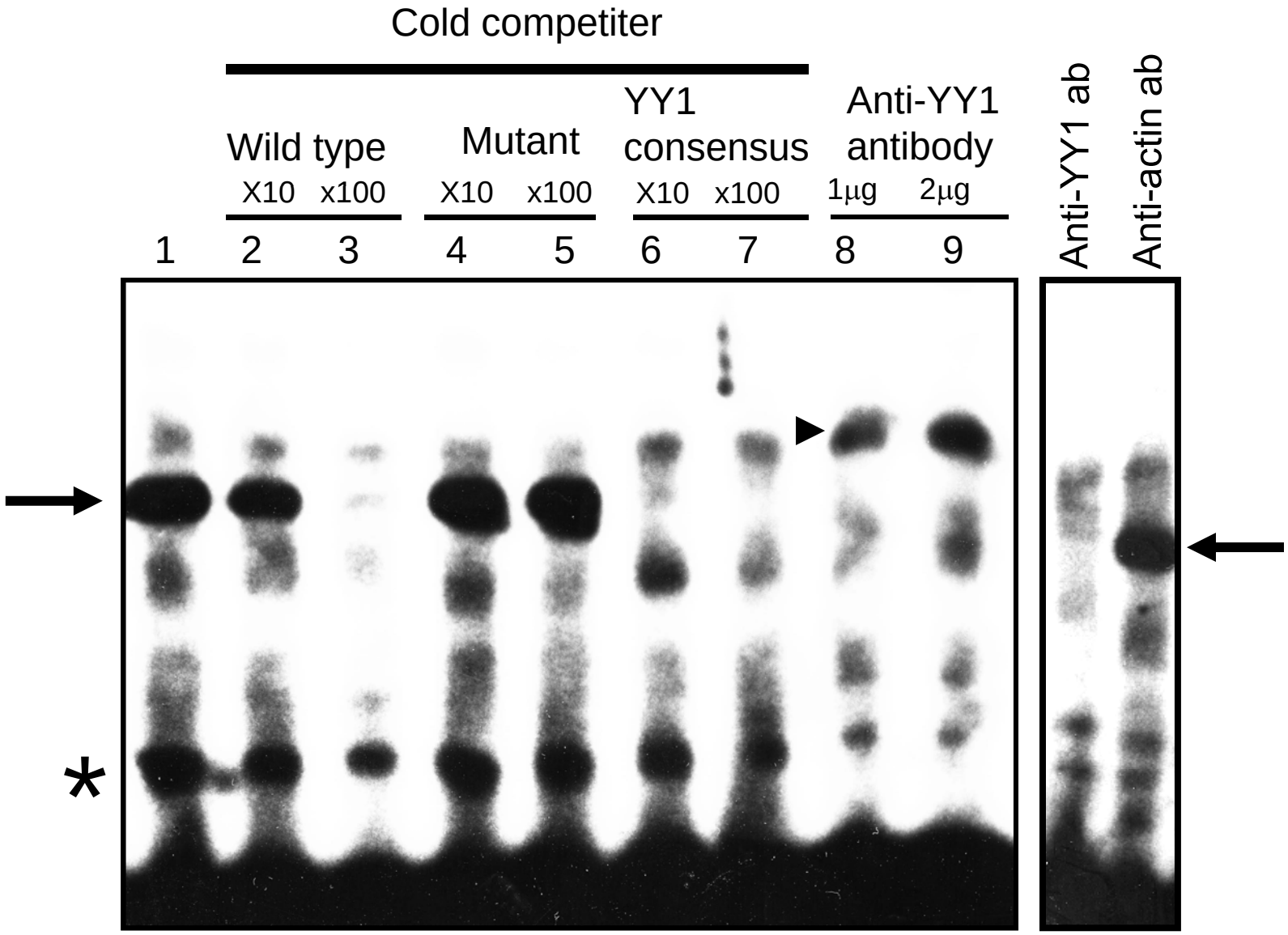
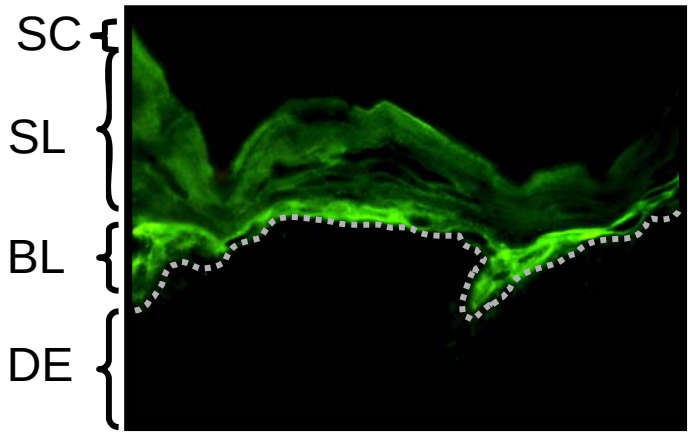

Loricrin Gene
BamHI
KpnI
Ex1
Intron I
Ex2
-1.5Luc
-0.5Luc
-0.3Luc
-0.5+1.1Luc
LUC
LUC
LUC
LUC
Fibroblasts
KC Low Ca
KC High Ca
0
100
200
Relative luciferase activity (x fold)


Exon1
Intron 1 (1.1kb)
Exon2
Sp1
PstI
EcoRV
NsiI
AP-1
+1
+318
+428
+692
+1070
+398
+458
WT
DEL1
DEL2
DEL3
DEL4
DEL5
LUC
Relative luciferace activity
25.1 ±2.34
22.6 ±2.53
57.1 ±3.33
52.1 ±2.79
53.8 ±2.54
28.1 ±1.65

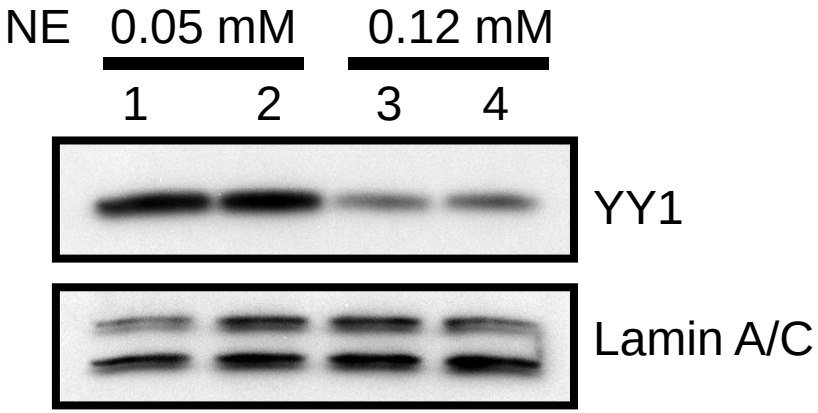

Relative luciferace activity
DEL5
LUC
28.1 ±1.65
+398 …. agtcataGAAATGGTGCtgatgg … +428
YY1
MUT/DEL5
LUC
46.7 ±2.03
+398 … agtcataGAAACAGTGCtgatgg … +428


Cold competiter
Wild type
X10
x100
Mutant
X10
x100
YY1 consensus
X10
x100
Anti-YY1 antibody
1μg
2μg
1
2
3
4
5
6
7
8
9
Anti-YY1 ab
Anti-actin ab
*

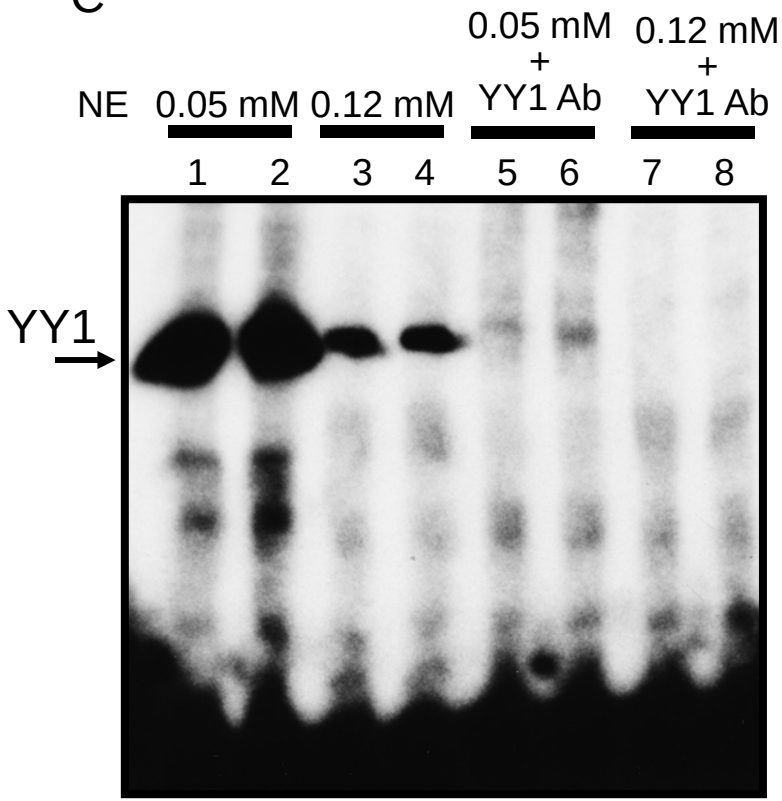
A

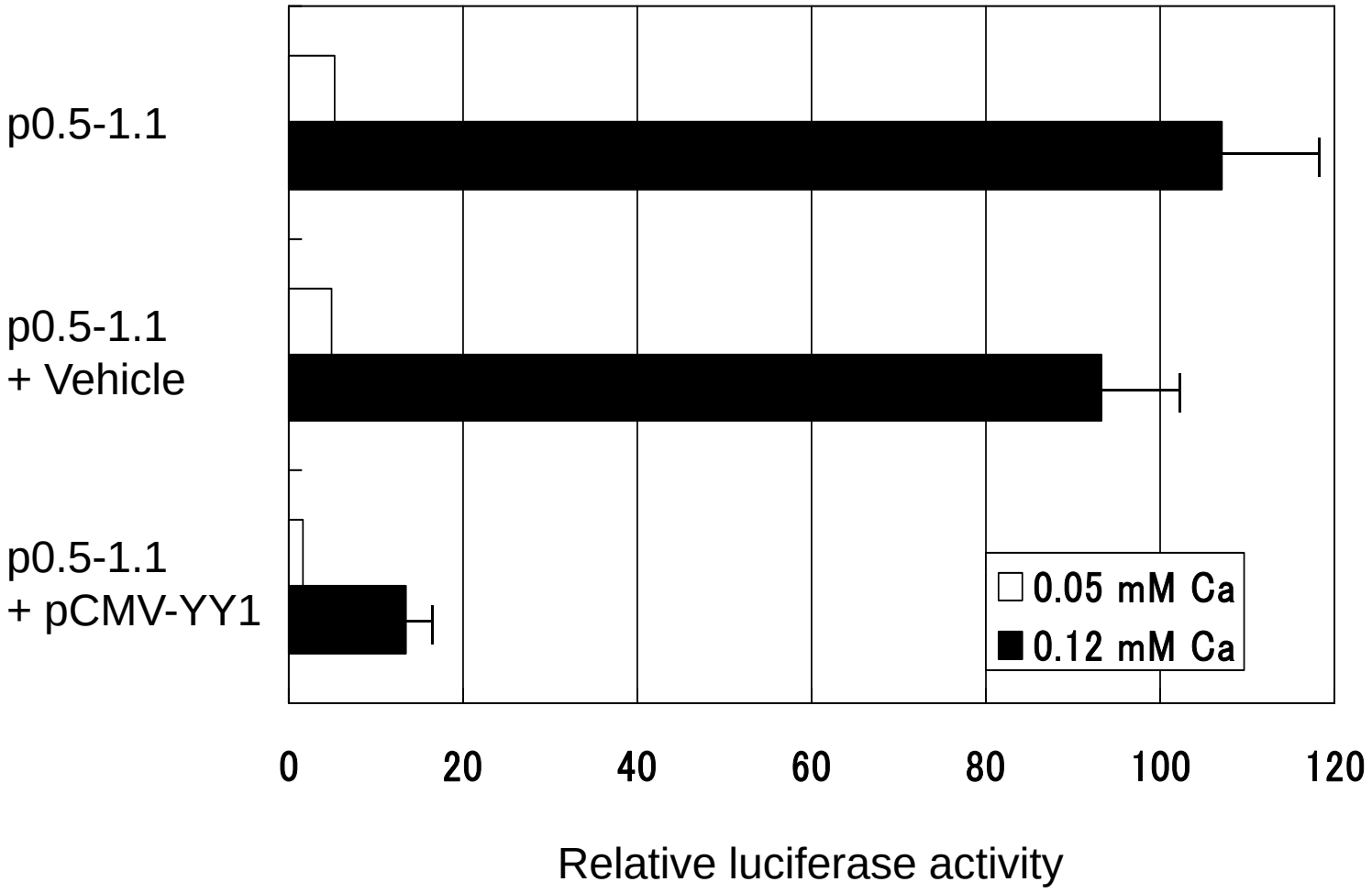

SC
SL
BL
DE

B

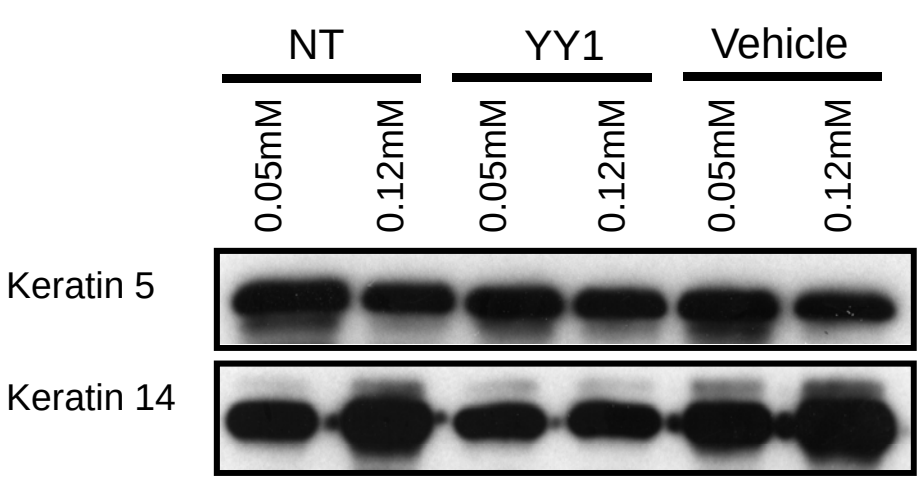

NE
0.05 mM
0.12 mM
1
2
3
4
YY1
Lamin A/C

C

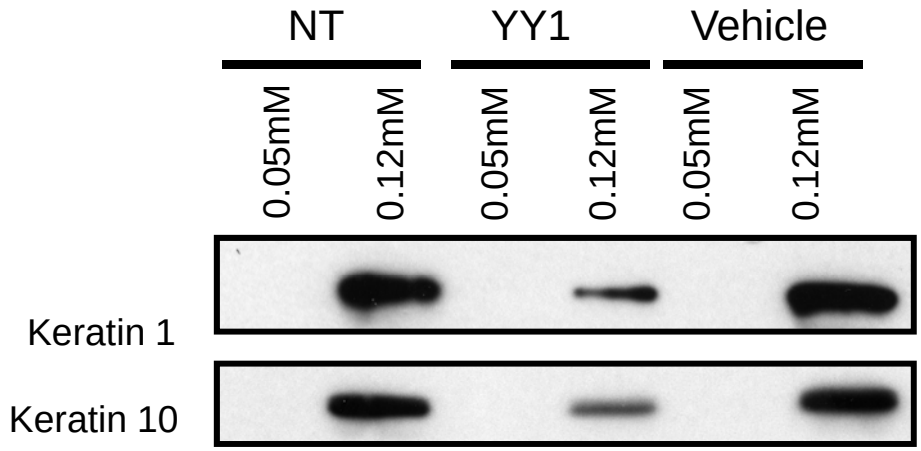

NE
0.05 mM
0.12 mM
0.05 mM + YY1 Ab
0.12 mM + YY1 Ab
1
2
3
4
5
6
7
8
YY1


p0.5-1.1
p0.5-1.1
+ Vehicle
p0.5-1.1
+ pCMV-YY1
0.05 mM Ca
0.12 mM Ca
0
20
40
60
80
100
120
Relative luciferase activity

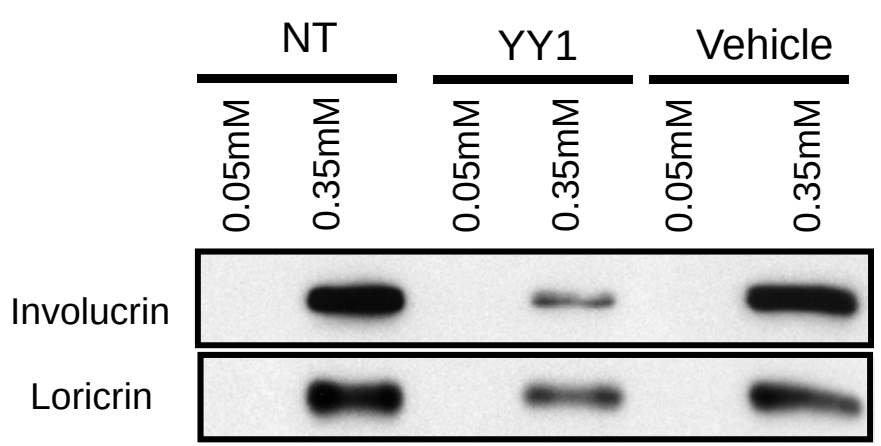
A


NT
YY1
Vehicle
0.05mM
0.12mM
0.05mM
0.12mM
0.05mM
0.12mM
Keratin 5
Keratin 14

B


NT
YY1
Vehicle
0.05mM
0.12mM
0.05mM
0.12mM
0.05mM
0.12mM
Keratin 1
Keratin 10

C


NT
YY1
Vehicle
0.05mM
0.35mM
0.05mM
0.35mM
0.05mM
0.35mM
Involucrin
Loricrin