Loricrin Expression in Cultured Human Keratinocytes Is Controlled by a Complex Interplay between Transcription Factors of the Sp1, CREB, AP1, and AP2 Families*

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The major protein component of the cornified cell envelope barrier structure of the epidermis is loricrin, and it is expressed late during terminal differentiation in epidermal keratinocytes. We have previously shown that an AP1 site located in the proximal promoter region (position −55) is essential for human loricrin promoter activity (Rossi, A., Jang, S-I., Ceci, R., Steinert, P. M., and Markova, N. G. (1998) J. Invest. Dermatol. 110, 34–40). In this study we show that its regulation requires complex cooperative and competitive interactions between multiple transcription factors in keratinocytes located in different compartments of the epidermis. We show that as few as 154 base pairs of 5′-upstream sequences from the cap site can direct the keratinocyte-specific expression in cultured keratinocytes. Mutation and DNA-protein analyses show that Sp1, c-Jun, an unidentified regulator, and the co-activator p300/CREB-binding protein up-regulate whereas Sp3, CREB-1/CREMα/ATF-1, Jun B, and an AP2-like protein (termed the keratinocyte-specific repressor-1 (KSR-1)) suppress loricrin promoter activity. We show that CREB protein can compete with c-Jun for the AP1 site and repress loricrin promoter activity. We show here that the protein kinase A pathway can activate loricrin expression by manipulation of the Sp1, Sp3, and KSR-1 levels in the nucleus. Thus, in undifferentiated cells, loricrin expression is suppressed by Jun B, Sp3, and KSR-1 proteins. But in advanced differentiated cells, levels of Sp3, KSR-1, and CREB proteins are lower; the unidentified regulator protein can bind; Sp1 and c-Jun are increased; and then p300/CBP is recruited. Together, these events allow loricrin transcription to proceed. Indeed, the synergistic effects of the Sp1, c-Jun, and p300 factors indicate that p300/CBP might act as bridge to form an active transcription complex.

The epidermis provides an essential protective barrier against the environment. As a stratified squamous epithelium, some inner basal cells become committed to terminal differentiation and move outward to form spinous, granular, and eventually fully differentiated, dead cornified layers. During this migration and differentiation program, unique sets of biochemical markers including specific structural proteins, such as keratins, their associated proteins, and processing enzymes, are synthesized (1–3). Another characteristic feature is the formation of the cornified cell envelope (CE), which is crucial for barrier function of the epidermis (4). The CE is a highly insoluble structure and contains a complex mixture of specific proteins such as involucrin, loricrin, small proline-rich proteins, XP-5 family members, cystatin A, elafin, S100 family members, and lipids that are covalently cross-linked by transglutaminases (5, 6). The quantitatively major CE protein in normal epidermis is loricrin (6, 10). However, the loricrin-deficient mouse model shows only minor defects in barrier function (11), probably because other CE proteins such as small proline-rich proteins, XP-5 proteins, and repeatin are up-regulated late during fetal development and apparently compensate for the loss of loricrin (12).

The expression of loricrin is strictly linked to keratinocyte terminal differentiation both in vivo and in vitro (13, 14). The loricrin transcripts are localized in the granular layers of the epidermis and some related orthokeratinizing epithelia (15, 16). Also, it has been demonstrated that loricrin transcription can be up-regulated when cultured keratinocytes are induced to differentiate by calcium (14). Thus, the tissue and differentiation specificity of loricrin expression make it an excellent model to study the sequences and transcription factors involved in the control of keratinocyte- and differentiation-specific gene expression. It has been reported that 6.5 kbp of sequences upstream of the cap site are required for the epithelium-specific expression of the mouse loricrin gene (17). Further study showed that a total of 14-kbp sequences are required to direct the differentiation-specific expression of mouse loricrin in transgenic mice (18).

Recent studies have shown that the regulation of epidermal gene expression appears to be controlled primarily at the transcription level. Several ubiquitously expressed transcription factors such as Sp1, NF-κB, AP2, AP1, Ets, and POU domain proteins, together with some unidentified factors (for example AP2-like factors), are involved in the specificity of epidermal gene expression. It is thought that the control of specificity of gene expression can be achieved through various arrangements of binding sites and the complex combinatorial interplay between these regulators (19–24).

The transcriptional regulation of the human loricin gene expression is poorly understood. In our previous study, we reported that a functional AP1 motif (located 55 bp upstream of the cap site) is indispensable for human loricin promoter ac-

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‡ The abbreviations used are: CE, cell envelope; CRE, cAMP-responsive element; PKA, protein kinase A; CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; CAT, chloramphenicol acetyltransferase; NHEK, normal human epidermal keratinocytes; NHF, normal human fibroblasts; KSSE, keratinocyte-specific silencing element; KSR-1, keratinocyte-specific repressor 1; HA, hemagglutinin; CMV, cytomegalovirus; TBS, Tris-buffered saline.
Complexity of Human Loricrin Transcription

NHEK and NHF transfections, cell cultures were washed once at 37°C with warm phosphate-buffered saline and then were preincubated at 37°C for 30 min with either Keratinocyte-SFM (Invitrogen) for NHEK, HaCaT, and NHF cells or Opti-MEM I medium (Invitrogen) for A-431, HeLa, HEK-293, COS-7, and NIH-3T3 cells. For each well, 1 µg of reporter plasmids and 0.5 µg of tk-β-gal plasmid were mixed with 6 µg of either FuGENE 6 or LipofectAMINE Plus in Keratinocyte-SFM and incubated for 20 min at 23°C. The lipid/DNA mixture was then added into each well and incubated for 16–18 h in the case of the NHEK, NHF, or HaCaT cells or 4–6 h for other cell types. At the end of the transfection period, the medium was replaced with the fresh medium in which the cells normally grow. For NHEK cells, unless indicated, all cultures were maintained in KGM-2 medium containing 1.2 mM calcium. NHF cells were grown and maintained in FGM-2 medium (Clonetics) supplemented with 2% fetal bovine serum. The lipid/DNA mixture was dissolved into cultures after the transfection was terminated. The control samples were treated with Me2SO (<0.1%, v/v) alone. Cells were harvested 48 h post-transfection. Cellular extracts were prepared as described by Pothier et al. (28). CAT assay, β-galactosidase assay, and total protein measurements were performed as previously described (23). The values for CAT were normalized by protein content and the β-galactosidase activity. Unless indicated, all of the data are the averages of at least three independent experiments with duplicated samples.

For co-transfection experiments, typically 1 µg of loricrin CAT constructs were co-transfected with 0.5 µg of expression vector as indicated. The corresponding parent vectors were used to ensure the same level of plasmid in all samples. All assays employed in this study include pRSV-c-Jun (from Dr. M. Karin) and c-Jun TAM under the control of cytomegalovirus promoter (a gift from Dr. M. Birrer). The Gal4/Sp1 expression vectors were used generously by Dr. R. Tjian (University of California, Berkeley, CA) and are pSG242 (parent vector), pSG-Sp1L1 (containing Sp1 amino acid residues 83–778, which include domains A–D), pSG-Sp1N (containing domains A–C, amino acid residues 83–621), pSG-Sp1AB (containing domains A and B, amino acid residues 83–542), and pSG-Sp1B (containing domain B, amino acid residues 263–542). All of these vectors contain cDNA encoding the Gal4 binding domain, amino acids 1–147 of the Gal4 protein, which were driven by an SV40 promoter (29). A-431, HaCaT, and NIH-3T3 cells selected for pCMV3-Sp3 were transiently cotransfected with Sp3 were removed, were gifts generously provided by Dr. D. Suske. The pCMV/β-gal300-CHA was kindly provided by Dr. S. Grossman and contains a pβ-gal200 cDNA insert from nucleotides 1134–8329 with a C-terminal HA tag cloned into the pCI vector. The expression plasmid of pSV-β-gal-CBP.HA.RK, pRSV-β-gal-CRE-B, and pRSV-β-gal-CRE-M was provided by Dr. R. Tjian. The pBS2AP2L and pBSV-AP2 were gifts from Dr. H. C. Birrer.

Gel Mobility Shift Assays—Nuclear extracts from NHEK, NHF, and HaCaT cells were prepared as previously described (23). The cytosolic fractions were obtained after the cells were homogenized with a tightly fitting glass homogenizer and centrifuged at 3000 × g in a refrigerated microcentrifuge for 10 min. Supernatants were used for gel shift assays. Forskolin-treated NHEK cells were grown in KGM-2 medium for 4 days and treated with forskolin (50 µM) for 6 h before harvesting. All buffers also contained phosphate inhibitors: sodium vanadate (200 µM), sodium fluoride (10 µM), and okadaic acid (2 µM). The double-stranded oligonucleotide probes were end-labeled with T4 polynucleotide kinase in the presence of γ-32P-ATP and then purified using DEAE cellulose. The SE gels (Invitrogen) were run in TBE buffer. For supershift experiments, the nuclear extracts were first incubated with 2 µg of the corresponding commercially available antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h at 4°C and then treated with the probe for another 30 min. The complexes were resolved on nondenaturing 6% polyacrylamide gels in 0.5× TBE buffer for 11 h at 4°C. After transfer, the membranes were washed once in 1× Tris-buffered saline (TBS), pH 7.5, and then incubated in blocking buffer (1× TBS, 0.1% Tween 20, 5% nonfat dry milk) for 1 h at 23°C. The membranes were washed three times for 5 min each with 1× TBS/T (1× TBS, 0.1%...
The cat constructs were transfected into NHEK cells as described under "Materials and Methods." The CAT activity was determined and normalized by β-galactosidase activity from the ptk-β-gal plasmid. Data are presented as CAT expression in -fold change over that from -45/+9 construct.

Twee 20) and then incubated with a 1:1000 dilution of either c-Jun or Sp3 polyclonal antibody (Santa Cruz Biotechnology) in blocking buffer Tween 20) and then incubated with a 1:2000 dilution of either c-Jun or Sp3 polyclonal antibody (Santa Cruz Biotechnology) in blocking buffer for 1 h at 23 °C. Following three washes with 1× TBS/T, signals were detected by using the SuperSignal West Pico Luminol/Enhancer kit (Pierce) and the manufacturer’s protocol prior to exposure to x-ray film.

RESULTS

Human Loricrin Promoter Activity in Cultured Cells—In a previous study, we reported that an AP1 motif located 55 bp upstream of the cap site (position -55) is essential for the activity of the loricrin promoter in NHEK cells (25). Using cultured keratinocytes (18) and a transgenic mouse approach, DiSepio et al. (17) showed that an AP1 site situated in the proximal promoter region is likewise critical for the expression of the mouse loricrin gene. However, it is highly unlikely that the tight control of the loricrin transcription is determined solely by interactions over the AP1 motif. Thus, the aim of the present study is to further elucidate the recognition sequences and their cognate binding factors that modulate the AP1-dependent activity of the human loricrin promoter. A series of fragments spanning increasing lengths of the 5’ regulatory region were cloned into the pCAT-Basic vector and their activities assessed after transfection into NHEK cells (Fig. 1). The highest activity was detected with construct -67/+9, which encompasses the loricrin TATA box and the AP1 site. In accordance with our previous results (25), deletion of the AP1 site caused a 10-fold reduction of expression. The addition of 45 bp of upstream sequences (-112/+9) reduced the CAT expression by 40%, 87 bp (construct -154/+9) reduced by 70%, and more sequences out to -450 reduced the expression to the level of the basal promoter (construct -45/+9, which encompasses only the TATA box). Such reductions of promoter activity could not be overcome by extension of the regulatory region to -1370. The loricrin constructs tested in this study gave similar results when NHEK cells were maintained under high calcium conditions (data not shown). Thus, this region does not encompass presumed calcium-responsive regulatory elements.

To assess whether these sequences functioned in a cell type-specific mode, the constructs were transfected into a variety of cells (Table I). The promoter activity of construct -45/+9 was comparable in all cell types (data not shown) and was about 15–20% of the level of the pCAT-Promoter construct containing the SV-40 early promoter (23). Inclusion of the AP1 site (construct -67/+9) resulted in a 9-fold enhancement in NHEK and HaCaT cells and about 5-fold enhancement in HeLa cells, which, although transformed, have preserved some features of their squamous cell origin (30). In contrast, this construct showed only basal promoter activity in simple epithelial and nonepithelial cells, consistent with the notion that the AP1 site is involved in cell type specificity of loricrin expression. Interestingly, mutation of the AP2-like site (keratinocyte-specific silencer element (KSS); see below) showed a 3-fold increase over that of construct -154/+9 for both NHEK and HaCaT cells but not in HeLa or other cell types. This suggests that the KSS site is involved in keratinocyte-specific repression of the loricrin promoter. These results also indicate that as few as 67 bp upstream of the transcription initiation site may be sufficient to restrict the activity of the human loricrin promoter to stratified squamous epithelial cells. Elements residing between -67 and -450 participate in the regulation of loricrin promoter activity and are further studied in detail here.

Identification of Transcription Factors That Bind on the Proximal Promoter Region of Human Loricrin—Initially, we focused our studies on the region between -67 and -154, which was responsible for a 2-fold repression of the loricrin proximal promoter. Comparison of these sequences with transcription factor databases (31) revealed an Sp1 site (position -125), an AP2-like site (-115), and a CRE-like motif (-100). Involvement of Sp1, AP2, and cyclic AMP response elements in transcriptional regulation in keratinocytes have been previously reported (32–34). Therefore, we first explored the ability of these motifs to interact with nuclear proteins using gel shift experiments. The specificity of the interaction was verified in competition experiments with unlabeled oligonucleotides carrying wild type or mutated sequences from this region or with di/dC oligomers.

A double-stranded oligonucleotide (-132/−90) encompassing the potential Sp1, AP2-like, and CRE-like sites (Fig. 2A) was labeled and incubated with nuclear extracts from NHEK cells. Four specific DNA-protein complexes (Fig. 2B, lane 1) were observed. Specific competition with unlabeled probe abolished complexes A, B, and D and reduced the intensity of complex C (Fig. 2B, lane 2). The origin of complexes A–D was determined in competition experiments with an array of oligomers in which three bp mutations had been created within the putative Sp1, AP2-like, and CRE-like recognition motifs. Mutant B1 competed out complexes A and B (lane 3). Mutants B2, B3, and B4, which affected the Sp1 motif, failed to interfere with the formation of complexes A and B (lanes 4–6). Mutants of the putative AP2-like motif (B4–B7) did not interfere with complex C (lanes 6–9). The competition for complex D was prevented by the mutants B1, B2, B4, B5, B6, and B11, where the CRE-like site was located (lanes 3, 4, 5, 6, 7, 8, and 13). Taken together, these results demonstrate that complexes A and B originate from interactions with the Sp1 site that and complex C was involved with the AP2-like site. Complex D appeared to be involved with all three recognition motifs.

To identify the proteins that participated in the above interactions, the binding reactions were performed in the presence of oligomers containing the corresponding recognition motifs or with corresponding specific antibodies. The formation of complexes A and B was abolished in the presence of an excess of unlabeled Sp1 consensus oligonucleotide (Fig. 2C, lane 3), confirming that both complexes resulted from the interactions with Sp1 proteins at the Sp1 site. Accordingly, the binding reactions were performed in the presence of antibodies against Sp1, Sp2, Sp3, and Sp4 transcription factors, or against transcription factor GKLF, which served as an unrelated antibody.
control. None of these antibodies affected the mobility or intensity of complexes C and D. The antibody against Sp1 supershifted complex A (arrowhead b, lane 4), whereas complex B was supershifted by the antibody against Sp3 (arrowhead a, lane 6). Since the antibodies against Sp2 (lane 5) and Sp4 (lane 7) did not result in a supershift, it is unlikely that these family members are present in either complex. Together, these results confirmed that interactions of Sp1 and Sp3 transcription factors over the sequence AGGTGGGGC were responsible for the formation of complexes A and B, respectively.

Likewise, the mutation scanning experiments determined that the potential AP2 recognition sequence GGCCGACAGGC is involved in the interactions resulting in the formation of both complexes C and D. Interestingly, when a shorter oligonucleotide (−117/−105), which encompasses only the AP2-like site, was used for competition (Fig. 2D, lane 3), both complexes C and D were competed out, suggesting that the AP2-like motif is responsible for the formation of both complexes. We could not design experiments to determine whether these two complexes were formed by two different proteins or whether the faster migrating band resulted from the degradation of binding proteins.

The identity of the proteins in complexes C and D were explored in interference experiments with antibodies against the known AP2 family members α, β, and γ. The intensity of complex C was reduced but not competed out in the presence of antibodies against AP2-β and AP2-γ but not AP2-α (Fig. 2D, lanes 4–6, respectively). None of the other three complexes (A, B, and D) was affected by these AP2 antibodies.

Since the antibodies against Sp1 and AP2 proteins were not able to interfere with complex D formation (Fig. 2, C and D) and CRE-like site mutant oligonucleotides failed to compete for complex D (Fig. 2B, lanes 11 and 12), we suspected that complex D might involve members of the CREB family of transcription factors. Accordingly, we performed the binding reactions of oligonucleotide −132/−90 in the presence of antibodies against a number of CREB proteins but could not detect any interference. We reasoned that the strong interactions between the Sp1 site and the high level of endogenous Sp1 proteins might mask protein binding over the CRE element. Indeed, when the binding reactions were performed with an oligonucleotide that encompassed only the AP2- and CRE-like motifs (−120/−90), in addition to the AP2-containing complex C, two relatively weak complexes were observed (Fig. 2E, lane 1) that were successfully competed by an excess of unlabeled probe (lane 2). Antibodies against CREB-1, CREM-1, and ATF-1 reduced the intensity of the upper band of the doublet (lanes 3, 5, and 6), and the supershifted bands were observed from both CREB-1

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**FIG. 2. Gel shift analyses on the loricrin promoter region.** A, the sequences of probes used for gel shift assays (−132/−90 for A–D; −120/−90 for E). The oligonucleotides (−117/−105, flanked by XbaI III and XbaI linkers) and mutant variants (B1–B11) were used for competition assays (see “Materials and Methods”). The Sp1, AP2-like, and CRE-like sites are indicated. B, the binding profile of oligonucleotides (−132/−90, lane 1) with NHEK cell nuclear extracts (NE) and poly(dI/ dC). Competition assays were carried with a 100-fold molar excess of unlabeled probe and mutation B1 to B11 (lanes 2–13) oligonucleotides. The arrows denote the specific complexes formed. C, competition with consensus Sp1 oligomer (lane 3, TGAATTCATCGGGGCGGGGCGAGC). Supershift assays are shown for complexes A and B with antibodies specific for Sp1 (lane 4), Sp2 (lane 5), Sp3 (lane 6), Sp4 (lane 7), and control GKLF (lane 8). The arrowheads denote the supershift caused by Sp1 (b) and Sp3 (a) antibodies. D, complexes C and D were competed out by the −117/−105 oligonucleotide (lane 3). E, the effect of gel shift by the antibody against CREB-1 (lane 2), CREM-1 (lane 3), ATF-1 (lane 5), ATF-2 (lane 6), and ATF-3 (lane 8). The two arrows denote the specific complexes; the arrowhead represents the supershifted bands.
and ATF-1 antibodies (Fig. 2E, arrowhead). The identity of the protein that forms the second weak band was not clear. We have tried the antibody against the phosphorylated CREB protein, but no change in binding profile was observed (data not shown). One possibility may be the presence of an unknown member of the CREB family in keratinocytes, but this has not been investigated further. Together, these data demonstrated that the CRE-like site is capable of interacting with the members of the CREB family present in keratinocytes.

Functional Analyses of the Human Loricrin Promoter—We next examined the role of Sp1, AP2-like, and AP1 motifs in the control of the loricrin proximal promoter. A series of mutated loricrin CAT constructs were prepared and transfected into NHEK and HeLa cells.

As shown in Fig. 3, mutation of the Sp1 site (construct A) showed about 75 and 55% decrease of the CAT activity in NHEK and HeLa cells, respectively, suggesting that interactions over this motif positively regulate the loricrin promoter in both cell types. However, this motif alone is not sufficient for the keratinocyte-specific expression of loricrin. When the AP2-like site was mutated (construct B), the expression level of CAT increased more than 2.5-fold compared with wild type in NHEK cells, but no effect was observed in HeLa cells. We also observed similar activation in HaCaT cells when the AP2-like site was mutated (data not shown). This suggests that the AP2-like site plays a key role in the keratinocyte-specific repression on the loricrin promoter activity. Hence, we termed this AP2-like element the keratinocyte-specific silencer element (KSSE) and the protein that binds to it the keratinocyte-specific repressor-1 (KSR-1). The mutation of the CRE-like site (construct C) only has little effect in both cell types. The effect of mutation of the AP1 site (construct D) was similar to that of the Sp1 site in both HeLa and NHEK cells. However, double mutations of the Sp1 and KSSE sites (construct E), KSSE and CRE-like sites (construct F), or KSSE and AP1 sites (construct G) reduced the promoter activity by 85, 40, and 80%, respectively, compared with the activity from construct B in NHEK cells. For HeLa cells, there is only a slight change (about 5–10%) in CAT activity for construct E, F, and G when compared with construct B. Taken together, the data show that Sp1, CRE-like, and AP1 elements play a positive role, whereas the KSSE motif is involved in a keratinocyte-specific repression on loricrin promoter activity. Furthermore, the CRE-like site only showed its positive effect when the KSSE site was mutated, suggesting that the presence of KSR-1 binding on the KSSE site might interfere with the binding of the regulator on CRE-like site.

Sp1 Activates and Sp3 Represses Human Loricrin Promoter Activity in NHEK Cells—The role of Sp1 as a transcriptional activator and Sp3 as a transcriptional repressor have been well documented (35, 36). To further confirm their involvement in the control of human loricrin promoter activity, the pRSV-Sp1 and pCMV-Sp3 expression vectors were co-transfected with wild type and mutant −154/+9 constructs. As shown in Fig. 4A, in the presence of pRSV-Sp1 plasmid, the level of CAT activity of the −154/+9 construct increased 50% compared with the control. A similar increase was observed when the KSSE site was mutated (construct −154/+9(KSSE)). This modest increase can be expected because of the high level of endogenous Sp1 in keratinocytes (37). However, no regulatory effect from forced expression of Sp1 was observed when the Sp1 site was mutated. To explore the role of Sp3 in the control of loricrin expression, loricrin CAT constructs were co-transfected with the pCMV-Sp3 expression vector. Forced expression of Sp3 repressed CAT expression of the −154/+9 construct more than 50% compared with the control (Fig. 4A, blank). Forced expression of Sp3 also suppressed CAT expression even when the KSSE site was mutated, indicating that Sp3 alone is sufficient to exert the repression effect on the loricrin promoter. When the loricrin constructs were co-transfected with the fingerless Sp3 expression vector (pCMV-Sp3/DBD), which lacked the DNA binding domain, CAT activity levels were similar to the control (Fig. 4A, hatched bar). This suggests that Sp3 requires its DNA binding domain to exert suppression on the loricrin promoter. No effect from forced expression of Sp1 or Sp3 was observed when the Sp1 site of the loricrin promoter was mutated. Furthermore, when equal amounts of Sp1 and Sp3 expression vectors were introduced together into NHEK cells with the loricrin constructs, Sp3 did not inhibit the CAT activity induced by Sp1 on −154/+9 and −154/+9(KSSE) constructs (Fig. 4B). Since NHEK cells have relatively low levels of Sp3 compared with Sp1 (Fig. 2B, lane 1), in an attempt to modulate the Sp1/Sp3 ratio, we transfected various amounts (0–6 μg) of pCMV-Sp3 into NHEK cells and evaluated the effects on loricrin promoter activities. Western blot analysis showed increasing level of Sp3 (Fig. 4C). The c-Jun protein was detected in parallel to serve as a loading control. Co-transfection of loricrin CAT construct with various amounts of pCMV-Sp3 cDNA showed a dose-dependent decrease in loricrin promoter activity (Fig. 4D). Together, these results show that both Sp1 and Sp3 are capable of binding and most likely compete with each other for the Sp1 site on the loricrin promoter. Furthermore, these results also confirm that Sp1/Sp3 ratios are involved in the control of loricrin promoter activity.

To further identify which transcriptional activation domains of Sp1 were involved in the transactivation of the loricrin promoter, we tested several expression vectors encoding different Sp1 domains fused with Gal4 protein. To exclude the effect from the endogenous Sp1 on the loricrin promoter activity, both the Sp1 and KSSE sites were replaced with a Gal 4 binding motif. The KSSE site was also eliminated to release its repression effect on the loricrin promoter and to maintain the proper length of the proximal promoter region, since both sites are very close and overlap each other (see Fig. 2A). As shown in Fig. 4E, the activation domain B of Sp1 (pSG-Sp1/Bl) showed a 2-fold increase of reporter activity of construct −154/+9(Gal4) over that of the same construct co-transfected with pSG424 parent vector alone. The combined glutamine-rich domains A and B (pSG-Sp1/A+B) increased the promoter activity more than 6-fold. The addition of activation domain C (pSG-Sp1/N) enhanced the promoter activity by 3.5-fold. The addition of domain D (pSG-Sp1/Wt) showed only a slight further increase of transactivation activity when compared with that from pSG-
Sp1/N expression plasmid. Together, these data demonstrate that the transactivation domains A, B, and C of Sp1 play a major role in the up-regulation of the human loricrin promoter in keratinocytes.

Forskolin Up-regulates Loricrin Promoter Activity—Fig. 3 showed that the CRE-like site plays a positive role in the control of loricrin promoter activity. Since CREB family proteins were involved in the formation of the specific complexes at the CRE-like site (Fig. 2E), we next assessed whether activation of the PKA signal pathway can lead to activation of human loricrin promoter. Various loricrin CAT constructs were transfected into NHEK cells, and the CAT activity was measured in the absence or presence of forskolin, an activator of the PKA pathway (38). As shown in Fig. 5, compared with the activity of untreated cells, the CAT activities of the −154/+9 construct showed 50% increase in cells treated with forskolin under high calcium medium. Little effect of forskolin was observed when the CRE-like site was either mutated (constructs −154/+9 (CRE), −154/+9(AP2/CRE)) or deleted (construct −67/+9). These findings suggest that the up-regulation of loricrin promoter activity by forskolin is exerted through the CRE-like site of the loricrin promoter.

The Effect of PKA, p300, CBP, and CREB Family Proteins on the Control of Loricrin Promoter Activity—Phosphorylation of CREB protein at Ser-133 is necessary for increasing its transcription activity (39), and this can be achieved by several kinases like protein kinase C, PKA, and calmodulin-dependent kinases (40–42). It has been reported that CREB exists as a phosphoprotein in differentiated keratinocytes (43). We next examined the role of CREB proteins in the control of loricrin expression and whether the effect was through the CRE-like site on the loricrin promoter. Expression plasmids of several members of the CREB family were used in co-transfection experiments. To our surprise, the expression of mouse mCREB-341 cDNA reduced the level of CAT expression of the −154/+9 loricrin construct by 70% (Fig. 6A). On the other hand, co-transfection of the dominant negative CREB expression vector (pRSV-KCREB) increased CAT expression by 80% above the control. Since the mutation carried by KCREB abolishes its binding to DNA, the KCREB/CREB heterodimers should be...
cultures were maintained either in high calcium (1.2 mM, or treated with forskolin (10 μM, hatched) under high calcium condition for 48 h before harvest. Data are presented as relative CAT activity in -fold change as described in the legend to Fig. 1.

transcriptionally inactive. Thus, our data suggest that mCREB-341 acts as a repressor for loricrin promoter activity. Further, the KCREB protein apparently can dimerize with endogenous CREB protein and release the repression. Also, both mCREMα and ATF-1 expression vectors reduced the CAT activity to the same level as mCREB-341, suggesting that all three are negative regulators for the loricrin promoter (Fig. 6A). Since all CREB-1, ATF-1, and mCREMα antibodies caused supershifted bands at the CRE-like site (Fig. 2), and co-transfection of all three expression vectors showed similar degrees of repression on loricrin, we have chosen to report only the findings observed with mCREB-341.

It is well known that phosphorylation of CREB allows for recruitment of the coactivators p300 and CBP (44–46). Since treatment with forskolin increased, whereas mCREB-341 repressed the loricrin promoter activity, we next explored the role of the catalytic subunit of the PKA, p300, and CBP to modulate the expression of the -154/+9 loricrin construct. In the presence of the PKA α subunit, the CAT activity increased more than 2-fold (Fig. 6A). This confirmed the observation from the forskolin experiments. The presence of CPB and p300 also showed an increase of CAT expression by 3- and 5-fold, respectively. Interestingly, after co-transfection of expression plasmids of either CBP and mCREB-341 or p300 and mCREB-341 together with the loricrin construct, the CAT activities were reduced to a similar level as for the same loricrin construct co-transfected with mCREB-341 alone. These results suggest that the presence of mCREB-341 compromised the activation of the loricrin -154/+9 construct by CBP and p300.

CREB Represses Loricrin Promoter Activity through the AP1 Site—The above experiments showed that forced expression of PKA α subunit, CBP, and p300 up-regulated but that mCREB-341 down-regulated loricrin promoter activity. Additional experiments were done to clarify these apparently contradictory observations. Deleted loricrin CAT constructs were used to examine where mCREB-341 displayed its repression effect on the loricrin promoter in NHEK cells. As shown in Fig. 6B, with the exception of the -45/+9 construct, mCREB-1 reduced the level of CAT activity to less than 50% in all loricrin CAT constructs compared with each respective control. The presence of KCREB showed opposite effects compared with mCREB-341 on constructs -154/+9, -112/+9, and -67/+9. No effect by KCREB was observed on constructs -154/+9(CRE) and -45/+9.

The negative effect by mCREB-341 on loricrin promoter activity is contradictory to the observation from both mutation (Fig. 3) and forskolin (Fig. 5) experiments where the CRE-like site appears to serve as a positive element for loricrin promoter activity. Furthermore, the repression by mCREB-341 and the enhancement by forskolin on the -67/+9 construct (Fig. 6B), which contained only the AP1 site and TATA box without any cryptic CRE site, prompted us to investigate whether mCREB-341 exerts its repression by interfering with the binding of AP1 proteins at the AP1 site. The ability of CREB proteins to form heterodimers with AP1 factors has been reported (47–49). Also, it has been documented that CREB can bind at an AP1 site and repress the AP1 reporter activity (43). First, to see if endogenous CREB dimerized with AP1 factors, the antibody against CREB protein was used in supershift analyses, but we did not see any interference of AP1 complex formation (data not shown). Second, we examined whether mCREB-341 competes with AP1 transcription factors for the AP1 site on the loricrin promoter. As shown in Fig. 6C, co-transfection of the pRSV-c-Jun expression vector increased the CAT activity of the -67/+9 construct more than 4-fold over the control. However, such up-regulation by c-Jun was abolished in the presence of the mCREB-341 expression plasmid. On the other hand, in the presence of mCREB-341, the increased amounts of pRSV-c-Jun failed to overcome the repression. Thus, these results demonstrate that mCREB-341 exerted its suppression through the AP1 site and prevented the binding of c-Jun. These results further support the observation that forced expression of KCREB increased loricrin promoter activity, most likely through the dimerization with endogenous CREB protein, and thereby releases suppression (Fig. 6B).

As shown in the supershift analyses, the CREB proteins bind on the CRE-like site (Fig. 2E). We next examined whether the CREB protein binds on the CRE-like site and exerted its effect on the loricrin promoter. To prevent the binding of CREB on the AP1 site, the AP1 site was mutated in the -154/+9 construct. The forced expression of mCREB-341 protein did not enhance but slightly reduced the CAT activity of -154/ +9(AP1) construct (Fig. 6D, white bar). One possibility is that the binding of KSR-1 on the KSSE site interferes with the binding of mCREB-341 on the CRE-like site. However, when the KSSE site was mutated, mCREB-341 still showed a repression effect. After double mutations either on the KSSE and CRE-like sites or on the KSSE and AP1 sites, mCREB-341 reduced more than 50% of CAT activity compared with the control (black bar). The mCREB-341 did not suppress only when both CRE-like and AP1 sites were mutated. Furthermore, it is known that CREB acts as a repressor in the hypophosphorylated state (50). Thus, when we tested the co-transfection of mCREB-341 with loricrin constructs in the presence of forskolin, we still observed the repression on the loricrin promoter by mCREB-341 (data not shown). On the other hand, the presence of pKCREB expression vector (Fig. 6D, hatched bar) increased the CAT expression compared with the control in all loricrin constructs except when CRE and AP1 sites were both mutated. Taken together, these data indicate the presence of other unidentified CREB family members that can up-regulate loricrin promoter in keratinocytes. Clearly, however, these results demonstrate that mCREB-341 acts as a repressor on loricrin promoter through two mechanisms; it can either compete with c-Jun for the binding on AP1 site, or it can dimerize with the unidentified endogenous regulator and interfere the CRE site-mediated transcription regulation.
Gel Shift Analysis Displays the Translocation of Sp1, Sp3, and KSR-1 Proteins by Forskolin—In an attempt to understand the possible mechanism for the up-regulation by forskolin of loricrin promoter activity, both cytosolic fractions and nuclear extracts from keratinocyte cultures treated with or without forskolin were prepared for gel shift analysis. As shown in Fig. 7A, whereas the intensity of the Sp1 complex was comparable between forskolin-treated and untreated samples, the intensities of the Sp3 and KSR-1 complexes were both less in the forskolin-treated samples than in the untreated control (compare lanes 1 and 6). Also, the complex D band was diminished in the forskolin-treated sample. The treatment of forskolin might either affect the DNA binding capability of Sp3 and KSR-1, or cause the translocation of proteins from the nucleus to cytoplasmic compartments. The latter indeed proved to be the case. When the same probe was incubated with the cytoplasmic fraction from forskolin-treated and untreated samples (Fig. 7B), two binding complexes (KSR-1 and D), which were competed out by −117/−105 oligonucleotides (lane 5), were found in untreated samples. In contrast, the complexes of Sp1, Sp3, and KSR-1 were found in the cytosol fractions of forskolin-treated samples. In addition, the −117/−105 oligomer reduced the intensity of the KSR-1 complex and competed out the complex D (lane 10). These results suggest that activation of the PKA signal pathway by forskolin can lead to the translocation of Sp1, Sp3, and KSR-1 proteins (for complexes C and D) from the nucleus to the cytoplasm. It therefore seems likely that this departure of especially Sp3 and KSR-1 results in the release of the suppression of loricrin expression, leading to the activation of the loricrin promoter. Furthermore, an increase of calcium concentration may not be involved in this process, since similar results were observed for forskolin-treated NHEK cells maintained either in low or high calcium medium (data not shown).

The Interplay of Sp1, c-Jun, and p300 on Loricrin Promoter Activity—The role of transcriptional cofactor p300 involved in the activation of Jun proteins (51) and the collaboration with Sp1 in the activation of the p21 promoter in HeLa cells (52) has been documented. Accordingly, we next explored the role of
p300 in modulating the activity of Sp1 and c-Jun on the loricrin promoter in NHEK cells. Loricrin constructs were co-transfected with vectors expressing either Sp1, p300, c-Jun, or c-Jun/TAM (a dominant negative mutant of c-Jun with a truncated transactivation domain). Co-transfection of Sp1 had a modest effect on the activity of the loricrin promoter. Forced expression of Sp1 did not show any effect when the KSSE site was mutated (Fig. 8A). Since the level of increase by c-Jun or p300 on the wild type construct is 80% higher than that of construct −154/+9 (KSSE) co-transfected with parent vectors (shaded bar); thus, forced expression of c-Jun or p300 is able to overcome the silencing effect and activate the loricrin promoter. Simultaneous co-transfection of c-Jun and Sp1 or c-Jun and p300 expression vectors synergistically increased the level of CAT activity 8-fold higher than the control. However, Sp1 and p300 together did not show a synergistic effect on the wild type construct. In fact, the level of expression in the presence of Sp1 and p300 was similar to that by p300 alone. Interestingly, when the KSSE site was mutated, co-transfection of Sp1 and p300 exerted a synergistic effect and activated the CAT expression to the same level as by c-Jun and p300 together (shaded bar).
than 16-fold, indicating that Sp1 can potentiate the synergistic effect caused by both p300 and c-Jun on the loricrin promoter. The transactivation effect of c-Jun was abolished in all cases when the transactivation domain of c-Jun was mutated (c-Jun/TAM). Also, the transactivation activity by c-Jun, Sp1, and p300 were either significantly reduced or abolished by mutations on either the Sp1 or AP1 sites, suggesting that both sites are indispensable for loricrin promoter activity (data not shown). Furthermore, Sp1, c-Jun, and p300 showed a dose-dependent activation on the loricrin promoter. Both c-Jun and p300 showed a higher dose responsiveness than that of Sp1 (Fig. 8B). These results support the observation from the co-transfection experiments that both c-Jun and p300 play a critical role in the control of loricrin expression. Taken together, these data show that the effect of p300 on loricrin expression could be exerted through the modulation of the c-Jun transactivation activity. Sp1 can exert its positive effect in the presence of c-Jun, suggesting that the two might interact directly or indirectly on the control of loricrin expression. The possible interplay between Sp1 and p300 can be achieved only when the KSSE site is mutated. Furthermore, these results also demonstrate that the high level of loricrin promoter activity can be achieved when Sp1, c-Jun, and p300 factors are present in NHEK cells.

DISCUSSION

In the present study, we have characterized the regulatory elements that are involved in cell type-specific expression of the human loricrin gene. Our study demonstrates that the transcription of this gene depends upon both proximal promoter and distal regions. The regulatory elements that are necessary to restrict the loricrin expression during the late epidermal differentiation lie beyond the first 1.3 kbp 5'-upstream from the transcription start site. However, we describe in detail here the presence and function of keratinocyte-specific elements within the first 154 bp upstream from the transcription initiation site.

The AP1 Motif—A comparison of the similar proximal promoter region of the human and mouse loricin genes reveals a common AP1 motif. However, different family members of AP1 proteins are involved in the two species. We have previously reported that c-Jun/c-Fos and Jun D activate and Jun B down-regulates human loricrin promoter activity (25). On the other hand, c-Fos/Jun B heterodimers activate, whereas c-Jun and Jun D show no effect on mouse loricrin promoter activity (18). Rutberg et al. (53) also reported that c-Fos/Jun B heterodimers induce AP1 transcription activity in mouse keratinocytes. One possible explanation as to why there are differences between humans and mice is that the expression pattern of AP1 family proteins is different in the epidermis of humans and mice. Indeed, Jun B, Jun D and Fra-2 are predominantly expressed in basal and suprabasal layers, whereas c-Jun, c-Fos, and Fra-1 are expressed in spinous and/or granular layer of human epidermis (54). However, in mouse epidermis, c-Jun is expressed in the basal layer, Jun D and Fra-1 are found in basal and spinous layers, and Jun B and Fra-2 are present in basal and granular layers (55). Thus, depending upon the availability of AP1 protein factors in the granular layer of the epidermis, the dimerization among different members of the AP1 family might lead to different regulatory effects on loricrin expression in human or mouse epidermis.

Furthermore, it is likely that the interactions between AP1 protein factors and other regulatory proteins in the proximal promoter region contribute to the control of cell type specificity of loricrin expression. For example, we have found here that in addition to the AP1 site, functional Sp1, KSSR, and CRE-like motifs are also present in the proximal human loricrin promoter, whereas only functional AP1 and Sp1 sites were reported in the mouse loricrin proximal promoter region (18, 56). Further, we found that the AP1 site alone is not sufficient to confer keratinocyte-specific expression to the human loricrin promoter, which is unlike another late differentiation marker profilaggrin in which the AP1 motif is enough to restrict the expression to epidermal cells (23). Thus, our data have shown that at least these four elements together contribute both activation and repression in a keratinocyte-specific manner to loricrin expression in cultured NHEK cells.

Competitive Interrelationship between Sp1 and Sp3 Proteins—The involvement of Sp1 and Sp3 proteins has been demonstrated in the regulation of several epidermally expressed keratin genes (20, 34, 57). This study on the human loricrin promoter is the first report that both Sp1 and Sp3 regulate a late epidermal differentiation marker. In general, Sp1 acts as an activator and Sp3 can act as either an activator or a repressor for transcriptional regulation. Since both recognize a similar GC-box motif, their relative availability during keratinocyte differentiation becomes critical (i.e. the balance between the levels of Sp1 and Sp3 may be responsible, at least in part, for the activation or repression on their target genes in the epidermis). Indeed, changes in the ratios of Sp1 and Sp3 have been reported to modulate promoter activity in other cell types (58). Further, one study reported that the level of Sp1 is up-regulated and the level of Sp3 is down-regulated when human keratinocytes are induced to differentiate by raising the extracellular calcium (59). Significantly, the present study confirms that the ratio of Sp1/Sp3 affects the loricrin promoter activity in NHEK cells (Fig. 4, B–D). On the other hand, when the PKA signal pathway was activated by forskolin, we found that both Sp1 and Sp3 were translocated from the nucleus to the cytosol (Fig. 7B), which will lead to changes in the relative ratios of Sp1 and Sp3 within nucleus. Because there is a relatively higher level of Sp1 compared with Sp3 in nuclear extracts of NHEK cells (Fig. 2B), it is therefore likely that Sp1 competitively replaces Sp3 binding, and this in turn activates the loricrin promoter. However, further work is needed to explore the nature of the nuclear-cytosolic translocation and to address whether phosphorylation might be involved.

Since Sp1 is a ubiquitously expressed transcription factor, it is possible that Sp1 interacts with other regulators to confer keratinocyte-specific gene expression. The involvement of Sp1 in the keratinocyte-specific expression of the human KRT14 gene has been demonstrated (24). We found that forced expression of Sp1 alone showed only modest effects on human loricrin promoter activity. However, when the Sp1 site was mutated, the promoter activity was reduced to the basal level (Fig. 3), indicating its critical role in the transcriptional control of human loricrin gene. By using the Gal4-Sp1 expression plasmids, we identified that the transactivation domains A, B, and C of Sp1 protein contribute the transactivation. Furthermore, our data showed that Sp1 and c-Jun act synergistically to increase loricrin promoter activity. A similar observation was reported in the study of the involucrin promoter in which the Sp1 site could enhance the activation by AP1 (60). Further study also indicated that Sp1 and AP1 motifs together are essential for involucrin expression during late keratinocyte differentiation (61). Besides the AP1 regulator, it has been reported that p300 is required for the induction of p21 expression in keratinocyte differentiation and that Sp1 and p300 might interact indirectly for the activation of the p21 promoter (52). In this study on the human loricrin promoter, we found a synergistic effect between Sp1 and p300 only when the KSSE site was eliminated, indicating that the putative KSR-1 protein might interfere with the interaction between Sp1 and p300. These data indicate a possible mutually exclusive binding between Sp1 and KSR-1.
The AP2-like (KSSE) Motifs—The involvement of the AP2 transcription factor in the regulation of tissue-specific expression of K1, K5, and K14 has been reported (19, 62, 64). In addition, several functional AP2-like response elements were found in the promoters of keratinocyte-specific genes such as bullous pemphigoid antigen (65), transglutaminase 1 (66), and involucrin (67). These AP2-like elements serve as enhancers and are believed to be involved in tissue-specific and differentiation-dependent expression. We have identified a KSSE site in the human loricrin promoter that has a similar recognition sequence (GCCGACAGGC) as the generic AP2 motif but instead is critical for keratinocyte-specific repression (Table I). In addition, we identified the presence of the putative KSR-1 protein, but its identity remains unknown. KSR-1 may not belong to the AP2 transcription factor family, since 1) forced expression of known AP2 family proteins activated the loricrin promoter (data not shown) and 2) KSR-1 was found to be a silencer not an activator of loricrin promoter activity. KSR-1 is located in both the cytoplasm and nucleus in keratinocytes (Fig. 7). Activation of PKA signal pathway leads to the relocation of KSR-1 through an unknown mechanism. It is not clear whether KSR-1 protein interacts with Sp3 directly or indirectly and together contributes the silencing effect on loricrin expression. However, forced expression of Sp3 reduced the promoter activity to a similar degree regardless of the presence of KSSE site (Fig. 4A). This suggests that Sp3 works independently for repression. Further work is needed to determine whether the KSR-1 protein is related to other proteins such as KTF-1 (65) and KDF-1 (67) that bind to AP2-like response elements found in the promoter region of keratinocyte-specific genes.

The CRE-like Motif—One intriguing finding in this study is the involvement of CREB family proteins in the regulation of loricrin transcription in keratinocytes. We found that CREB-1, CREMs, and ATF-1 proteins act as inhibitors of the loricrin promoter. It has been documented that CREB and AP1 proteins can form heterodimers and bind to AP1 sites (48, 68, 69). The involvement of AP1 family members in the transcription regulation of epidermal genes has been well documented (23, 63), but there are very few studies on the role of CREB proteins. In the human small proline-rich protein 1 promoter region, a CRE-like site (~588) was identified, but no detailed study was conducted on its role in the regulation of small proline-rich protein 1 expression (32). Jessen et al. (33) reported that the functional AP1 and CRE motifs were involved in the regulation of human transglutaminase 1 gene expression, but the possible interaction between the two was not studied. Rutberg et al. (43) demonstrated that CREB family members are capable of binding to AP1 sites and repress the transcription activity of a AP1 reporter in differentiated mouse keratinocytes in vitro.

Our new work on the human loricrin promoter is the first epidermal gene in which there is a clear interaction between CREB and AP1 proteins at the AP1 site. Our data demonstrated that CREB proteins suppress the AP1-mediated activation of the loricrin promoter by binding on the AP1 site (Fig. 6C). Although we did not detect any change by CREB-1 antibody on the AP1 binding profile in the supershift assays, the possible dimerization between CREB and AP1 factors in keratinocytes cannot be ruled out. Since forced expressed c-Jun alone can overcome the repression of ~154/+9 construct (Fig. 8A), it is possible that the high levels of introduced c-Jun can compete with the endogenous CREB protein for the binding on AP1 site and activate loricrin promoter.

Interestingly, a CRE-like site is located just 40 bp downstream from the AP1 site and 10 bp upstream of the KSSE site. This CRE-like site serves as a positive element only when the KSSE motif is mutated, suggesting the likelihood of mutually exclusive binding between the proteins for these two sites. The possible existence of the unidentified positive regulator that can bind on the CRE-like site is based on two lines of evidence: 1) there is a faster moving band of specific doublet complex, as shown in Fig. 2E, and 2) when the AP1 and KSSE sites were mutated, mCREB-341 still repressed the promoter activity, yet in the presence of KCReB, the promoter activity increased over the control. The effects from both mCREB-341 and KCReB diminished only when both CRE and AP1 sites were mutated (Fig. 6D). One likely possibility is that the CREB protein dimerizes with the unidentified positive regulator, which binds on this CRE-like site. This should result in suppression of the loricrin promoter activity in NHEK cells. Thus, the CREB protein can either bind on the AP1 site or dimerize with AP1 proteins and/or with an unidentified regulator, leading to the suppression of loricrin promoter activity. The distribution pattern of the members of CREB family in the skin of newborn mice has been reported (43). The CREB, CREMs, and ATF-1 are abundant in nuclei in both basal and spinous layers but not in granular layers. Accordingly, it is likely that the CREB protein represses loricrin promoter activity, whereas the keratinocytes are in basal and spinous layers but not in the granular layer, where c-Jun levels are high. Thus loricrin expression can be initiated.

Model for the Control of the Human Loricrin Promoter—We propose the following model to account for our new data (Fig. 9). When NHEK cells are in the basal and spinous layers, the loricrin promoter activity is suppressed through 1) the interactions of Jun B, Sp3, and KSR-1 proteins by binding to their
respective motifs, which prevents the binding of Sp1 and c-Jun (Fig. 9A); or 2) the CREB proteins bind on the AP1 site and/or dimerize with other positive regulators (Fig. 9B). When keratinocytes move to the granular layer a number of changes occur: the Sp1/Sp3 ratio changes due to the departure of Sp3 and KSR-1 proteins from the nucleus; the levels of CREB proteins become lower; relative levels of Sp1 and c-Jun become higher; and p300/CBP is recruited. Thus, a cooperative interaction among these factors results in the assembly of basal transcription machinery over the initiation site and leads to the activation of human loricrin transcription (Fig. 9C). Thus, the precise coordination in the combinatorial interactions provides an on-off mechanism for optimal loricrin expression in the granular layer of human epidermis.

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