

Bleomycin Hydrolase Is Regulated Biphasically in a Differentiation- and Cytokine-dependent Manner

RELEVANCE TO ATOPIC DERMATITIS[§]

Received for publication, July 27, 2010, and in revised form, December 5, 2010. Published, JBC Papers in Press, December 29, 2010, DOI 10.1074/jbc.M110.169292

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Loss-of-function mutation in the profilaggrin gene is a major risk factor for atopic dermatitis (AD). Previously, we showed that a neutral cysteine protease, bleomycin hydrolase (BH), has a role in generating natural moisturizing factors, and calpain I is an upstream protease in the filaggrin degradation pathway. Here, we investigated the transcriptional regulatory mechanisms of BH and the relevance of BH to AD. First, we cloned the 5'-flanking region of BH. Deletion analyses identified a critical region for BH promoter activity within –216 bp upstream. Electrophoretic mobility shift assay revealed that MZF-1, Sp-1, and interferon regulatory factor-1/2 could bind to this region *in vitro*. Moreover, site-directed mutagenesis of the MZF-1 and Sp-1 motifs markedly reduced BH promoter activity. These data indicate that BH expression is up-regulated via MZF-1 and Sp-1. Interestingly, a Th1 cytokine, IFN- γ , significantly reduced the expression of BH. Analyses with site-directed mutagenesis and small interference RNA supported the suppressing effect of IFN- γ on BH expression. On the other hand, a Th2 cytokine, IL-4, did not show any direct effect on BH expression. However, it down-regulated MZF-1 and Sp-1 in cultured keratinocytes, indicating that IL-4 could work as a suppressor in BH regulation. Lastly, we examined expression of BH in skins of patients with AD. BH activity and expression were markedly decreased in AD lesional skin, suggesting a defect of the filaggrin degradation pathway in AD. Our results suggest that BH transcription would be modulated during both differentiation and inflammation.

During terminal differentiation, keratinocytes synthesize characteristic proteins, including filaggrin, loricrin, trychohyalin, and involucrin, which are encoded by the so-called “epidermal differentiation complex,” a cluster of genes on human chromosome 1q21 (1, 2). Filaggrin is a key protein in formation of the cornified cell envelope, which is critical for an effective epidermal barrier (3, 4). Recent genetic studies have shown that loss-of-function mutations in *FLG*, the human gene encoding profilaggrin and filaggrin, are the cause of the semi-dominant skin scaling disorder ichthyosis vulgaris and are a major risk

factor for the development of atopic dermatitis (AD),² eczema-related asthma, and other allergic phenotypes (4–6). These mutations are present in 10–40% of the population, depending on race and country of habitation.

Filaggrin is deiminated by peptidylarginine deiminase, resulting in its unfolding and further degradation into hygroscopic amino acids that constitute the natural moisturizing factors (NMF) that maintain epidermal hydration (7, 8). NMF consist primarily of amino acids, including citrulline, and their derivatives, such as pyrrolidone carboxylic acid and urocanic acids, together with lactic acid, urea, citrate, and sugars (9–11). Previously, we identified the neutral cysteine protease bleomycin hydrolase (BH) as an NMF-generating enzyme and also showed that calpain I is an upstream protease in the filaggrin degradation pathway (12). BH inactivates the anti-tumor antibiotic bleomycin by hydrolyzing the amide bond in the β -aminoalanine moiety. Although BH is widely expressed in mammalian tissues (13–16), skin seems to contain the highest amount, and relatively high levels of BH were detected in the kidney and liver of 6-week-old male rats (16). BH exhibits mainly broad specificity aminopeptidase activity, with citrulline being the most effective substrate (12, 14). It is involved in homocysteine-thiolactone metabolism (17), antigen presentation (18, 19), and amyloid- β -peptide clearance (20, 21). Interestingly, BH knockout mouse shows analogous phenotypes to those of filaggrin-deficient flaky tail *ft/ft* mouse, including ichthyosis and atopy-like phenotype and tail constriction (22–24). These findings indicate that BH has a role in maintaining epidermal barrier function.

Nucleotide sequence analysis of the 5'-flanking region of the human BH gene revealed that it has characteristic features of a housekeeping gene (25). However, the regulatory mechanisms of human BH gene have not been investigated in detail, and the *cis*-elements and binding factors required for expression in keratinocytes are unknown. Here, we report the identification and characterization of the minimal promoter of BH gene and identification of *cis*-acting elements involved in the regulation of its expression. Recognition sequences for two differentiation-related factors and one inflammation-related transcription factor were localized just above the transcription start site.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S4.

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² The abbreviations used are: AD, atopic dermatitis; BH, bleomycin hydrolase; NMF, natural moisturizing factors; IRF, interferon regulatory factor.

Our results indicate that defects of the filaggrin degradation pathway play a role in the pathophysiology of AD.

EXPERIMENTAL PROCEDURES

Materials—Human IL-4 and IFN- γ were purchased from PEPROTECH EC (London, UK). Human IL-13 and IL-17A/F were from R & D Systems (Minneapolis, MN). Citrulline 4-methylcoumaryl-7-amide was obtained from Bachem Bioscience (Bubendorf, Switzerland). Anti-BH IgG was prepared according to the methods reported by Kamata *et al.* (16). All other chemicals used were of reagent grade.

Keratinocyte Cultures—Normal human epidermal keratinocytes derived from neonatal epidermis (Kurabo, Osaka, Japan) were cultured in EpiLife medium (Cascade Biologics, Portland, OR) containing calcium at a low concentration (0.03 mM) and HKGS growth supplement (Cascade Biologics). All of the cells were incubated at 37 °C with 5% CO₂ and used within four passages. Keratinocytes were harvested at 80% confluency, 100% confluency, 2 days after confluency, and 2 days after confluency in 2 mM calcium. To investigate the effects of cytokines on BH expression, IFN- γ , IL-4, IL-13, or IL-17A/F was added to the culture medium of proliferating cells as well as differentiated cells (2 days after confluency) and incubated for 24 h before RNA isolation.

Cloning of the 5'-Flanking Region of BH—Based on the nucleotide sequence of human BH gene (25), the 5'-flanking region was amplified with a Genome Walker kit (Clontech, Mountain View, CA) according to the manufacturer's instructions, using gene specific primer 1 (GSP1), 5'-TCCCTCGAGTCTGTAT-CAGAGCAGCTACA-3' and gene specific primer 2 (GSP2), 5'-TGAACACGCGTCCGAGCTGCTCATGGCG-3'. Briefly, the primary PCR was performed with GSP1 and adaptor primer 1 using a two-step PCR protocol recommended by the manufacturer: seven cycles at 94 °C for 25 s and 72 °C for 4 min followed by 32 cycles at 94 °C for 25 s and 67 °C for 4 min and final extension at 67 °C for 4 min, with *EX Taq* DNA polymerase (Takara, Shiga, Japan) in the presence of 5% dimethyl sulfoxide. The primary PCR mixture was then diluted and used as a template for secondary PCR amplification with GSP2 and adaptor primer 2. The secondary PCR was identical to the first, except for the use of five initial cycles instead of seven, followed by 20 cycles instead of 32. Sequential 5'-deletion mutants of the 5'-flanking region of BH were generated by PCR using the primers listed in supplemental Table S1. After amplification, all of the PCR products were cloned into *pGEM-T* Easy vector (Promega, Madison, WI) and sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

To construct the reporter plasmid *pGL3-1216/+1*, PCR was carried out using *pGEM-T-1216/+1* as a template and a pair of specific BH primers containing restriction sites for KpnI and MluI (5'-CCGGGTACCATCAGAGTTCCTTAGAA-3' and 5'-TAAATACGCGTTGGCGCCCACGCTGCCG-3') under the following conditions: initial denaturation at 94 °C for 4 min and 30 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 4 min. The PCR product obtained was digested with KpnI and MluI and cloned into the *pGL3-Basic* vector (Promega), which contains the firefly lucif-

erase gene. All of the constructs were prepared using a Qiagen plasmid midi kit (Qiagen).

Site-directed Mutagenesis—Mutagenesis of MZF-1, Sp-1, and IRF-1/2-binding sites was performed by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. For making deletion mutations in Sp-1, 5'-GGACCCCGTTTCAGCCT-CCCCGCC-3' (forward primer of mutant Sp-1 site) and 5'-GGCGGGGAGGCTGAAACGGGGTCC-3' (reverse primer of mutant Sp-1 site) were used. For MZF-1 mutant, 5'-GACTCAGCAACGCGGTTTTGTCCCTCCGC-3' (forward primer of mutant MZF-1 site) and 5'-GCGGAGGGACAAA-ACCGCGTTGCTGAGTCA-3' (reverse primer of mutant MZF-1 site) were used. For IRF-1/2 mutant, 5'-GCCGCCG-AGCCTCCGGCGCTCC-3' (forward primer of mutant IRF-1/2 site) and 5'-GGAGCGCCGGAGGCTCGGCGGC-3' (reverse primer of IRF-1/2 site) were used.

Transfection and Measurement of Promoter Activity—Keratinocytes were cultured on a 12-well tissue culture plate at a density of 5×10^4 cells/well and transfected with 1 μ g of each construct using FuGene HD Transfection reagent (Roche Applied Science). To correct for transfection efficiency, all of the cells were cotransfected with *pGL4.74* (hRluc-TK) vector, which contained the *Renilla* luciferase gene under the control of the HSV-TK promoter (Promega). Unless otherwise specified, the cells were harvested 24 h after transfection and lysed with 250 μ l/well of passive lysis buffer (Promega). Luciferase activities were analyzed with a dual luciferase reporter assay system (Promega) and an Autolumat plus luminometer (Berthold Technologies, Bad Wildbad, Germany). *Firefly* luciferase activity was normalized for *Renilla* luciferase activity. Three transfections were carried out independently for each construct, and the results were expressed as the mean values.

Quantitative Real Time RT-PCR Analysis—Transcription levels of BH and related factors were analyzed by quantitative real time RT-PCR. Total RNA was extracted from cultured cells with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The cDNA was reverse-transcribed with SuperScriptTM II (Invitrogen). Real time RT-PCR was performed on a LightCycler rapid thermal cycler system using a LightCycler 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer's instructions. Information on primers used is shown in supplemental Table S2. GAPDH was used as a reference gene. The specificity of amplified fragments was confirmed by means of quantitative analysis of the melting curve with LightCycler analysis software. Amounts of mRNA were normalized to those of GAPDH and finally presented as ratios to those of untreated control.

siRNA-based Suppression for Putative Transcription Factors—Cultured keratinocytes were transfected using Lipofectamine RNAi Max (Invitrogen) with 40 nM siMZF-1 and siSp-1 (Invitrogen), siIRF-1, siIRF-2, siGATA-1, and siControl A (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. The cells were cultured in antibiotic-free medium for 24 h, and then total RNA was extracted and analyzed by real time RT-PCR as described above.

EMSA—Double-stranded oligonucleotide probes were prepared by annealing the single-stranded biotinylated and nonla-

beled oligonucleotides (supplemental Table S3). Nuclear extraction and EMSA were performed using a nuclear extraction kit (Panomics, Santa Clara, CA) and LightShift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL). The nuclear extracts (5 μ g) were incubated with 1 $\times$ binding buffer, 50 mM KCl, 5 mM MgCl₂, 5 mM EDTA, 2.5% glycerol, and 50 ng of poly(dI-dC) and biotinylated probe (25 fmol) corresponding to the MZF-1, Sp-1, IRF-1/2, and GATA-1-binding sites for 20 min at room temperature. For competition assay, a 200-fold excess of unlabeled probe was added to the binding reaction prior to the addition of the biotinylated probe. These incubation mixtures were then electrophoresed in 8% polyacrylamide gel with 0.5 $\times$ TBE buffer and transferred to Biodyne B nylon membrane (Pall, Port Washington, NY). The bands were visualized with the LightShift Chemiluminescent EMSA kit.

Immunohistochemistry—Human skin specimens were obtained, with informed consent, from patients with atopic dermatitis at Tokyo Medical University. The study was approved by the Institutional Review Board of Tokyo Medical University and the Shiseido Committee on Human Ethics. All of the skin samples were fixed with 4% paraformaldehyde and embedded in paraffin. Human atopic dermatitis (lesional and nonlesional skin) and normal skin sections were incubated with anti-rat BH IgG and monoclonal anti-human filaggrin IgG (Harbor Bio-Products, Norwood, MA) for 1 h at room temperature, then washed with PBS, and further incubated with fluorescence-conjugated secondary antibodies, Alexa Fluor 555 or 488 (Molecular Probes Inc., Eugene, OR). As a control, normal mouse IgG was used instead of primary antibody. DAPI (Molecular Probes) was used to visualize nuclei. Immunohistochemical localization of BH and filaggrin in normal and AD skin was observed by using confocal microscopy (LSM5 PASCAL; Carl Zeiss Japan). The lens was Plan APOCHROMAT 20 $\times$ magnification.

Western Blot Analysis—SDS-PAGE for tape-stripped extracts from normal and AD skin was done using 5–20% polyacrylamide gels (e-PAGEL E-T520L, ATTO) under reducing conditions. After electroblotting, the Immobilon-P PVDF membrane (Millipore, MA) was stained with rabbit anti-BH IgG, followed by ECL anti-rabbit IgG HRP-linked F(ab') fragment from donkey (GE Healthcare). BH was detected using an ECL plus Western blotting detection system (GE Healthcare).

Measurement of BH Activity in Skin—Corneocytes were obtained by means of tape stripping from lesional and nonlesional skin of AD patients ($n = 18$) and from the inner side of the lower arm of healthy volunteers ($n = 30$). Cellophane tape (Nichiban, Tokyo, Japan) was firmly attached to the skin and repeatedly pressed with fingers over the entire area. After removal of the tape, a 12-cm² (2 $\times$ 6 cm) section was cut into small pieces and immersed in 1 ml of extraction buffer (0.1 M Tris-HCl, pH 8.0, containing 0.14 M NaCl and 0.1% Tween 20). The samples were sonicated three times for 20 s each, and cornified cell extract was obtained by means of centrifugation. Protein concentration was measured using a DC protein assay kit (Bio-Rad) according to the manufacturer's instructions. BH activity in the extract was measured at 37 $^{\circ}$ C for 30 min with extracts and 0.1 mM citrulline 4-methylcoumaryl-7-amide as a substrate in 0.1 M Tris-HCl, pH 7.5, containing 10 mM DTT

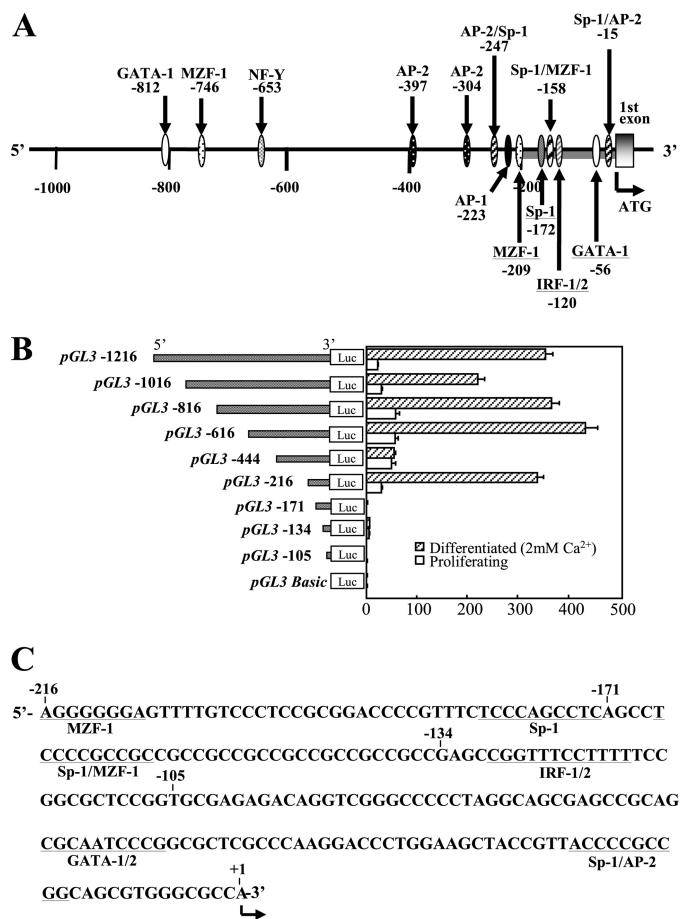


FIGURE 1. Identification of the minimal promoter region of BH. A, schematic illustration of the 5'-flanking region of BH. Potential transcriptional factor-binding sites predicted by the Genome Net MOTIF program (classification: vertebrates; cut-off score: 85%; Kyoto University Bioinformatics Center, Kyoto, Japan). B, identification of minimal promoter of BH. Luciferase activity was measured in proliferating or differentiated (+2 mM CaCl₂) keratinocytes transfected with expression plasmids containing sequentially deleted fragments of the 5'-flanking region of BH. The values were normalized for transfection efficiency by cotransfection with *Renilla* expression plasmid and expressed as the means $\pm$ S.D. of six independent experiments. C, sequence and putative transcription factor-binding sites of minimal promoter of BH. Numbering of the nucleotides begins with the transcription initiation site as +1. Putative transcriptional factor-binding sites are underlined.

and 5 mM EDTA. The fluorescence intensity was measured at 355 nm/460 nm using a Fluoroskan Ascent (Thermo Scientific).

Statistics—We employed post hoc analysis, Dunnett method. All of the statistical analyses were performed using KyPlot 5.0 (KyensLab, Tokyo, Japan).

RESULTS

Isolation and Characterization of Human BH Gene Promoter—A search with the Genome Net MOTIF program revealed numerous putative transcription factor-binding sites in the 5'-flanking region of human BH (Fig. 1A). In particular, within the -216/+1 region near the position of the transcriptional start site, there were sequences that closely matched the consensus sequences recognized by MZF1, Sp-1, IRF-1/2, and GATA-1/2, suggesting that these transcription factors are involved in regulation of BH promoter activity. The rate of homology in each site against consensus sequence was listed in supplemental Table S4.

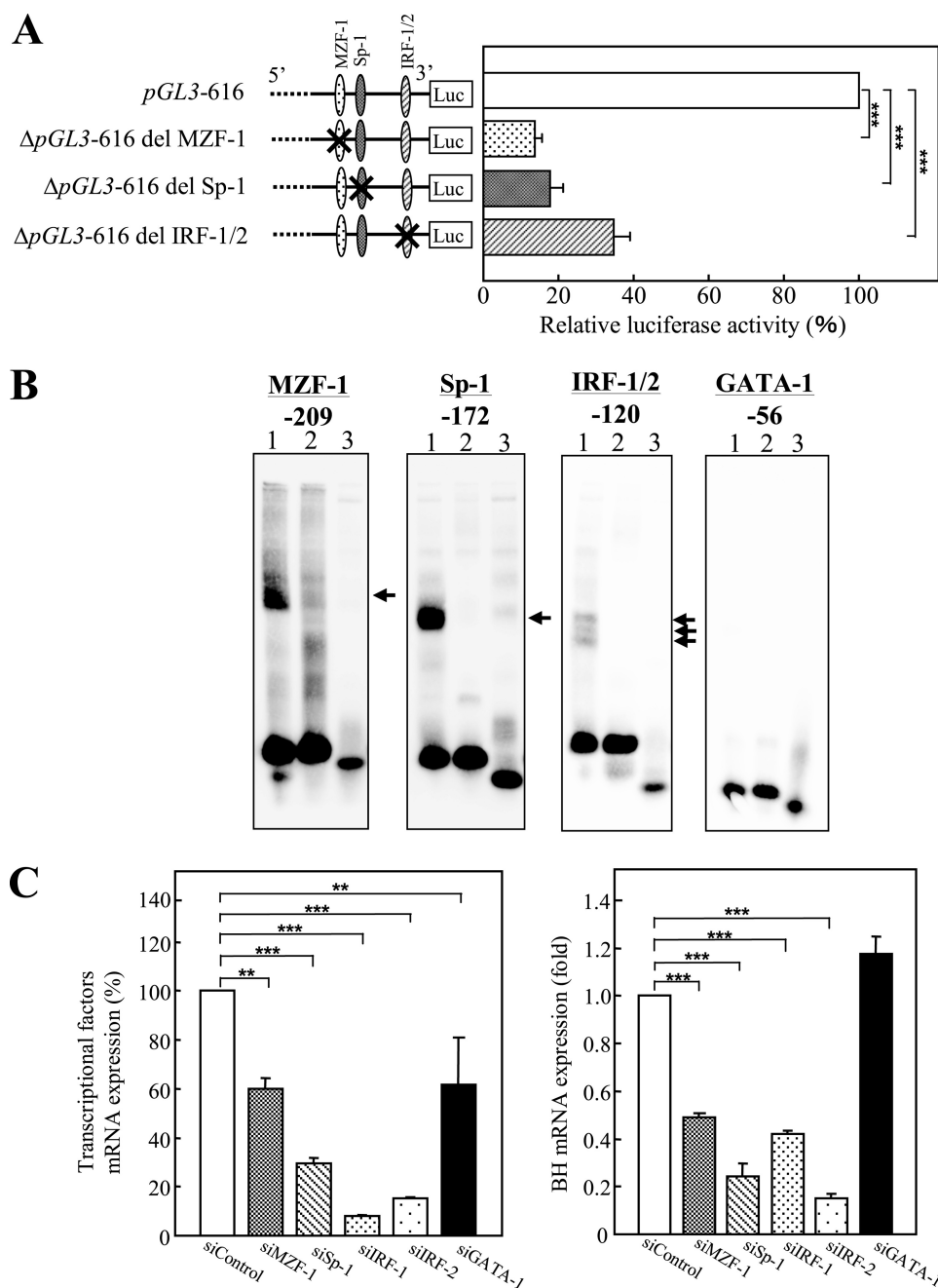


FIGURE 2. Characterization of the cis-elements in the BH promoter region. A, characterization of the transcription factor-binding sites in the BH promoter by site-directed mutagenesis. Schematic diagram of deletion constructs of the putative transcription factor-binding site and their luciferase activities in cultured keratinocytes are shown. Site-directed mutagenesis was carried out with the construct spanning from -616 to $+1$ nucleotides. The values were corrected for transfection efficiency by cotransfection with the *Renilla* expression plasmid and are expressed as the means $\pm$ S.D. for three independent experiments. ***, $p < 0.001$ (post hoc analysis, Dunnett method). B, binding of Sp-1, MZF-1, IRF-1/2, and GATA-1 to cis-acting elements of the BH promoter. EMSAs were performed using a synthetic oligonucleotide probes corresponding to regions containing putative transcription factor-binding sites and nuclear extracts ($5 \mu\text{g}$) of cultured keratinocytes. Sequences of the probes used are given in supplemental Table S3. The protein-DNA complexes were resolved on 6% polyacrylamide gels in 0.5% TBE buffer. Lanes 1, binding profile of the biotinylated probe with nuclear extracts, lanes 2, binding profile after competition with a 200-fold molar excess of unlabeled probe, lanes 3, binding profile of the biotinylated mutant probe. The arrows indicate shifted bands of biotinylated probes. C, effects of siRNA targeted to MZF-1, Sp-1, IRF-1, IRF-2, and GATA-1. Keratinocytes were transfected with each siRNA (40 nm) and cultured for 2 days after confluency in 2 mm calcium. Inhibition of transcription factors expression by respective siRNAs was confirmed by real time RT-PCR and compared with control siRNA (left panel). The effect of transcription factor silencing was also investigated (right panel). Note the decrease of BH mRNA after knockdown of MZF-1, Sp-1, IRF-1, and IRF-2. The data were normalized to the GAPDH gene. **, $p < 0.01$; ***, $p < 0.001$ (post hoc analysis, Dunnett method).

To define the promoter region of BH more closely, deletion analyses were carried out (Fig. 1B). The highest level of luciferase activity was detected in differentiated keratinocytes transfected with *pGL3-816*. However, relative luciferase activities of deletion plasmids remained high until the deletion proceeded

to *pGL3-216*. Among the constructs, the plasmid containing fragment $-444/+1$ (designated *pGL3-444*) showed significantly lower activity in cultured keratinocytes, suggesting the presence of an upstream suppressor activity in the $-616/-444$ region. These results demonstrated that the $-216/-1$ region

contained the minimal promoter for BH gene transcription, and its nucleotide sequence is shown in Fig. 1C. This sequence did not include a TATA or CCAAT box, suggesting the house-keeping nature of this gene (25). On the other hand, several transcriptional factor-binding sites, such as MZF-1, Sp-1, IRF-1/2, and GATA-1/2 were present in this core promoter region.

Identification of Potential *cis*-Elements Involved in BH Gene Regulation—To determine potential *cis*-elements of the minimal promoter involved in the transcriptional control of BH gene expression, we constructed a new series of deletion mutants, targeting each *cis*-element. When the binding sites of MZF-1, Sp-1, and IRF-1/2 were deleted, the promoter activity was markedly down-regulated (Fig. 2A).

Furthermore, we investigated whether these transcription factors can actually bind to each putative binding site. For this purpose, EMSA was performed with nuclear extracts from cultured keratinocytes and biotinylated double-stranded oligonucleotide probes containing a MZF-1-, Sp-1-, GATA-1-, or IRF-1/2-binding site. As shown Fig. 2B, Sp-1, MZF-1, and IRF1/2 bound to the corresponding target region of the BH promoter, whereas GATA-1/2 did not bind. Involvement of these transcription factors was further confirmed by suppressing their expression using siRNAs. Although suppressing effect varied depending on each siRNA, down-regulation of MZF-1, Sp-1, IRF-1, and IRF-2 significantly affected BH expression (Fig. 2C). However, suppression of GATA-1 did not show any inhibitory effect on BH expression. These were in accordance with EMSA results, indicating that the binding of MZF-1, Sp-1, and IRF-1/2 functionally regulates BH expression. These results also suggest that these binding sites of the promoter region between -216 to -105 bp are essential *cis*-elements for BH transcription.

Cytokine-mediated Regulation of BH Gene Expression—Because BH is an NMF-generating enzyme (12), it may be involved in the pathophysiology of AD. Thus, we examined the effects of Th1, Th2, and Th17 cytokines on BH gene expression. Fig. 3A showed that in proliferating keratinocytes, Th1 cytokine IFN- γ down-regulated BH mRNA expression in a dose-dependent manner. On the other hand, Th2 and Th17 cytokines did not show any significant effect on the expression of BH. Similar results were obtained with differentiated keratinocytes (data not shown). To elucidate the role of IFN- γ in the regulation of BH gene expression, we carried out promoter assay to identify cytokine-responsive elements. As shown in Fig. 3B, IFN- γ down-regulated BH promoter activity in cultured keratinocytes transfected with *pGL3*-BH-616, which contained the IRF-1/2-binding sequence between -131 and -120 . After deletion of this sequence, IFN- γ no longer suppressed the promoter activity (Fig. 3B). In addition, to determine whether IRF-1/2 is an essential mediator of IFN- γ -induced down-regulation of BH, we used siRNA to suppress IRF-1 and IRF-2 gene expression. The activity of IFN- γ was significantly suppressed in cultured keratinocytes transfected with either IRF-1 or -2 siRNA (40 nM) (Fig. 3C). These results strongly suggest that the IRF-1/2-binding sequence is essential for IFN- γ induced down-regulation of BH gene expression.

Expression of BH and Related Factors in Cultured Keratinocytes—To investigate the mechanisms of transcriptional regulation in the epidermis, we analyzed the expression

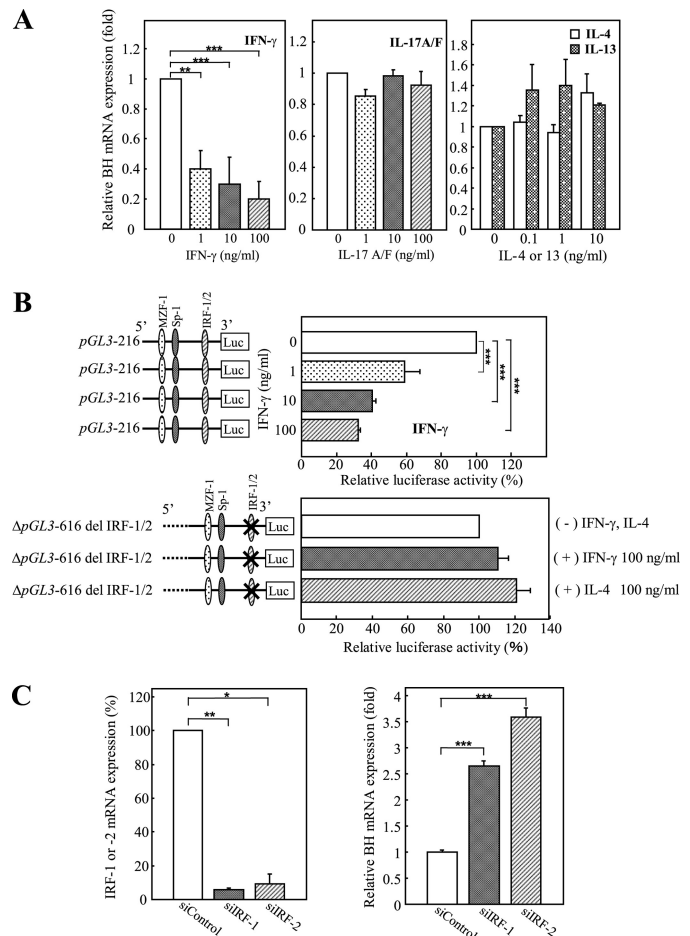


FIGURE 3. Effects of cytokine on the expression of BH in cultured keratinocytes. A, real time RT-PCR analysis of BH expression. IFN- γ , IL-4, IL-13, or IL-17A/F were added to the culture medium of proliferating cells, and incubation was continued for 24 h before RNA isolation. The data were normalized to the GAPDH gene. The results are the means $\pm$ S.D. for three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (post hoc analysis, Dunnett method). B, mutation analysis of IRF-1/2-binding site. Keratinocytes were transfected with *pGL3*-216 containing intact IRF-1/2-binding site of the BH promoter region and treated with IFN- γ for 24 h (upper panel). Keratinocytes transfected with Δ *pGL3*-616 (deletion mutant of IRF-1/2 site) were incubated with or without 100 ng/ml IFN- γ or IL-4 for 24 h (lower panel). Luciferase activity was measured as described under "Experimental Procedures," and the values were normalized for transfection efficiency by cotransfection with *Renilla* expression plasmid and expressed as the means $\pm$ S.D. of three independent experiments. ***, $p < 0.001$ (post hoc analysis, Dunnett method). C, effect of siRNA targeted to IRF-1/-2. The keratinocytes were transfected with IRF-1 or IRF-2 siRNA (40 nM) and cultured for 24 h, then treated with 10 ng/ml IFN- γ , and further cultured for 24 h before RNA isolation. Inhibition of IRF-1 or IRF-2 expression by their respective specific siRNAs was confirmed by real time RT-PCR as compared with control siRNA (left panel). The effect of IRF-1 and IRF-2 silencing was also investigated (right panel). Note the marked increase of BH mRNA after knockdown of IRF-1, as well as IRF-2. The data were normalized to the GAPDH gene. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (post hoc analysis, Dunnett method).

of BH, calpain I, and putative transcription factors in proliferating or differentiated cells by means of real time PCR. As shown in Fig. 4A, BH mRNA was up-regulated in differentiated keratinocytes, such as those at 2 days after confluency (3.6-fold) and those cultured at high calcium concentration (6.8-fold), compared with proliferating keratinocytes. These results are consistent with the promoter assay data (Fig. 1B). Similar results were obtained for calpain I (~ 2.5 -fold up-regulation). We also examined the expression pattern of various transcrip-

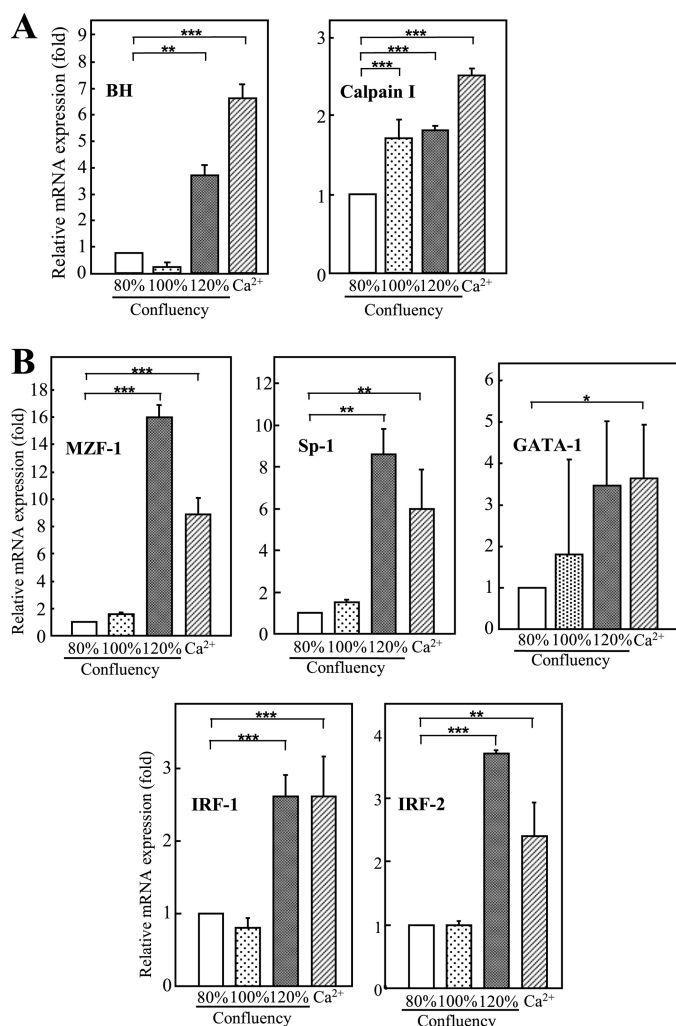


FIGURE 4. Expression of BH and related gene transcripts. A, expression of BH and calpain I gene transcripts in cultured keratinocytes. Keratinocytes were harvested at 80% confluency (80%), 100% confluency (100%), 2 days after confluency (120%), and 2 days after confluency in 2 mM calcium (Ca²⁺). The relative BH or calpain I mRNA levels were determined from cDNA samples by real time RT-PCR as described under "Experimental Procedures." The data were normalized to the GAPDH gene. The results are expressed as the means $\pm$ S.D. for three independent experiments. B, expression of MZF-1, Sp-1, GATA-1, IRF-1, and IRF-2 gene transcripts in cultured keratinocytes. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (post hoc analysis, Dunnett method).

tion factors, such as MZF-1, Sp-1, GATA-1, IRF-1, and IRF-2, in cultured keratinocytes. As shown in Fig. 4B, these transcription factors were up-regulated in differentiated keratinocytes, paralleling the expression of BH. However, GATA-1 mRNA expression was significantly lower ($< 1/32$), as compared with the other factors. It seems likely that GATA-1 does not play an important role in keratinocytes. Thus, we suggest that BH is synthesized in a differentiation-dependent manner via MZF-1 and Sp-1. The fact that IRF-1 and IRF-2 were also up-regulated by differentiation stimuli indicates that BH expression is highly responsive to IFN- γ .

Effects of Th1 and Th2 Cytokines on the Expression of Putative Transcription Factors—We further investigated cytokine-dependent modulation of these transcription factors. Fig. 5A shows that IFN- γ strongly up-regulated IRF-1 mRNA expression in a dose-dependent manner. Similarly, IRF-2 expression

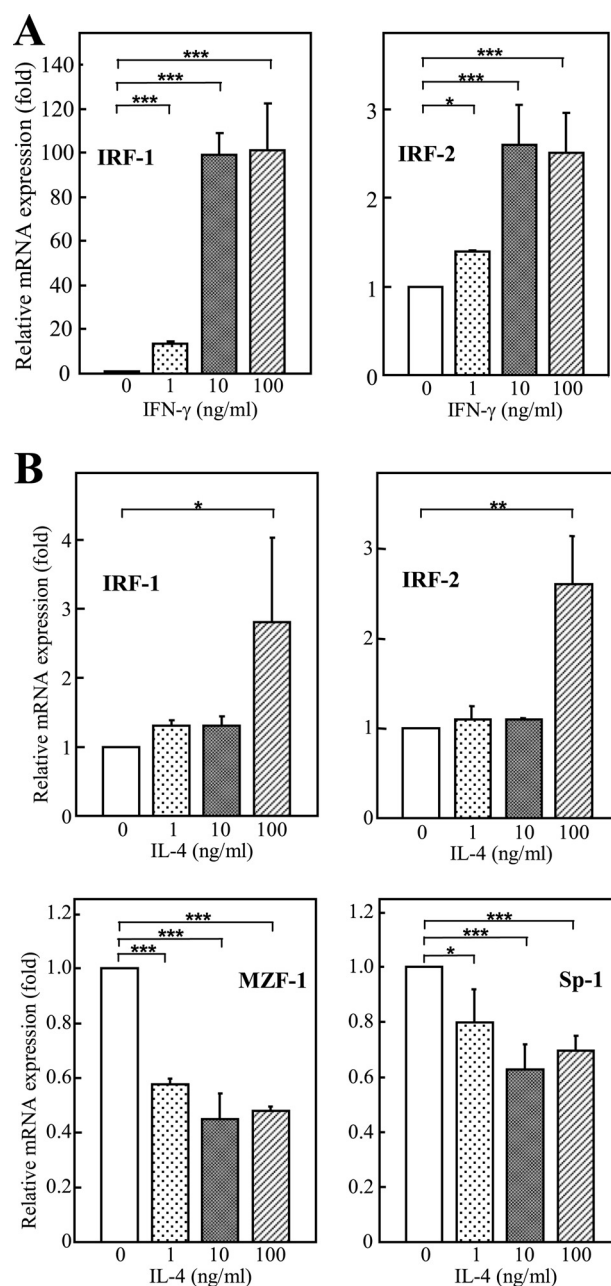


FIGURE 5. Effects of cytokines on the expression of various transcription factors. A, effect of IFN- γ on the expression of IRF-1 and IRF-2. B, effect of IL-4 on the expression of IRF-1, IRF-2, MZF-1, and Sp-1. IFN- γ or IL-4 was added to the culture medium of proliferating cells, and incubation was continued for 24 h before RNA isolation. Real time RT-PCR analysis was performed. The data were normalized to the GAPDH gene. The results are the means $\pm$ S.D. for three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (post hoc analysis, Dunnett method).

was up-regulated in the presence of IFN- γ . In contrast, expression of IRF-1 and IRF-2 was significantly increased in the presence of IL-4 only at 100 ng/ml (Fig. 5B). Interestingly, MZF-1 and Sp-1 were both down-regulated most effectively in the presence of IL-4 at 10 ng/ml (Fig. 5C). These results suggest that BH expression is regulated directly and indirectly by Th1 and Th2 cytokines, respectively.

BH Is Down-regulated in Atopic Dermatitis Skin—Although Palmer *et al.* (26) reported that loss-of-function mutations in *FLG* are associated with the pathogenesis of AD, not only gene

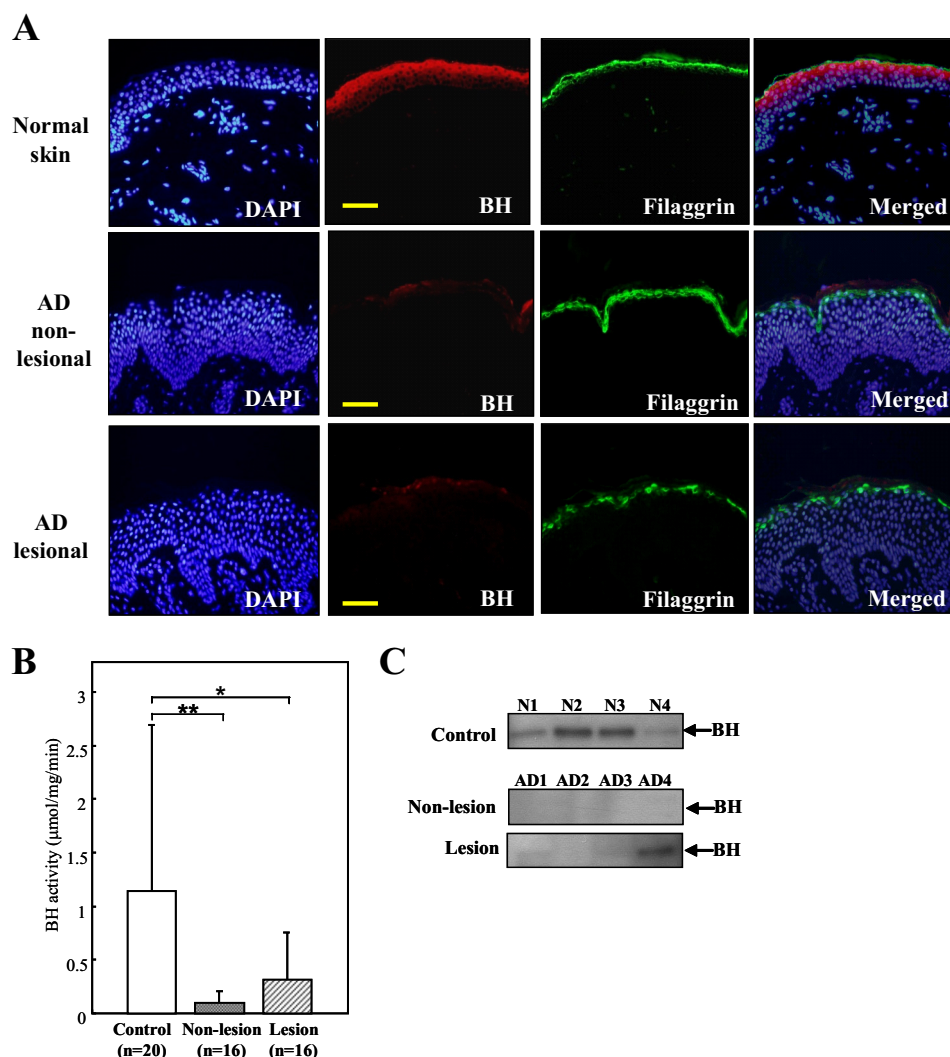


FIGURE 6. Changes of BH expression in AD. *A*, immunohistochemical analyses for BH (red) and filaggrin (green) in paraffin-embedded sections of nonlesional and lesional skin of patients with AD and normal skin were performed. The nuclei were counterstained with DAPI. Paraffin-embedded sections of human skin tissue after dual immunofluorescence labeling were subjected to confocal microscopy. Scale bars, 100 μm . *B*, BH activity of nonlesional and lesional skin of patients with AD. Tape-stripped samples were assayed for BH activity toward citrulline 4-methylcoumaryl-7-amide as described under "Experimental Procedures." $^*p < 0.05$; $^{**}p < 0.01$ (post hoc analysis, Dunnett method). *C*, Western blot analysis of BH in normal and AD skin. Each sample contained 0.8 μg of total protein extracted with tape-stripping corneocytes from normal, AD-nonlesional and AD-lesional skin. Four samples each from normal (N1–N4) and paired AD extracts (AD1–AD4) are shown.

defects, but also impairment of the degradation pathway might contribute to AD pathology. Therefore, we next examined the localization of BH and filaggrin and the BH activity in lesional and nonlesional skin of AD patients. In normal epidermis, double staining with anti-BH and anti-filaggrin antibodies showed colocalization of BH and filaggrin in the upper epidermis, especially in the granular layer, as reported earlier (Fig. 6A). Higher magnification clearly showed that BH was localized from the granular to the cornified layer, whereas filaggrin was restricted to the granular cells. In contrast, BH expression was dramatically decreased in lesional and nonlesional skin of AD patients examined in this study ($n = 7$). All of these patients showed relatively weak filaggrin staining, although significant staining was always detected (Fig. 6A). Nonimmune normal mouse IgG was used as a negative control and did not show any positive reaction (data not shown). In addition to immunohistochemistry, BH activity was measured in corneocyte extracts from tape-stripped

samples obtained from 18 AD patients and 30 healthy volunteers. Extracts from lesional and nonlesional skin of AD patients showed substantially decreased BH activity (to 27.1 and 8.8%, respectively), compared with that from healthy individuals (Fig. 6B). These results demonstrated that BH was colocalized with filaggrin, and its activity was dramatically decreased in the skin of patients with AD. This was also confirmed with Western blot analysis (Fig. 6C). Normal corneocyte extracts showed a clear BH protein band with 45 kDa in all of the samples. However, the band was hardly detectable in nonlesional samples, and only one patient showed a 45-kDa band in lesional samples.

DISCUSSION

In this study, the regulatory mechanisms of BH gene expression were studied by cloning and functional characterization of the promoter region. Promoter analysis identified a critical region for BH promoter activity within -216

bp upstream (Fig. 1B). In this region, putative MZF-1 and Sp-1-binding sites showed significant effects on BH promoter activity (Figs. 1C and 2A). Interestingly, it has been reported that Sp-1 and MZF-1 are also involved in the regulation of peptidylarginine deiminase 1 (27), a critical enzyme for initiation of filaggrin degradation. Sp-1 is the prototypical member of the Sp/Krüppel-like family of zinc finger proteins that function as transcription factors in mammalian cells (28, 29). It is thought to take part in virtually all facets of cellular function, including proliferation, apoptosis, differentiation, and neoplastic transformation (28, 29). In human epidermis, Sp-1 is an important regulator of genes participating in epidermal differentiation, including those of involucrin, loricrin, transglutaminase (30), and peptidylarginine deiminase 1, 2, and 3 (27, 31, 32). MZF-1 is a transcription factor belonging to the Krüppel family of zinc finger proteins and is expressed in totipotent hemopoietic cells, as well as in myeloid progenitors (33, 34). However, the function of MZF-1 in transcriptional regulation in mammalian epidermis has not been reported. We found simultaneous up-regulation of MZF-1 and Sp-1, as well as BH, in differentiated keratinocytes compared with proliferating keratinocytes (Fig. 4B), indicating a role of BH in differentiation, rather than a housekeeping role. Our results clearly showed that these transcription factors act as activators for the basic transcriptional regulation of BH during keratinocyte terminal differentiation.

On the other hand, investigation of *cis*-acting elements further defined the IRF-1/2-binding site in this region. Using EMSA, we confirmed direct binding of IRFs to the BH promoter region (Fig. 2B). Site-directed mutagenesis of this binding sequence caused a significant decrease of BH promoter activity (Fig. 2A). Therefore, IRF-1/2 transcriptional factor would also be required for the minimal promoter activity of the BH gene under basal conditions. The IRF family is a group of transcription factors, and so far, nine IRF members (IRF-1–9) have been identified in various cell types and tissues (35–38). These IRF molecules play roles in antiviral defense, immunoresponse/modulation, and cell growth regulation under stimulation with IFN- α , β , and γ (35–38). IRF-1 and -2 have been shown to act as an agonist-antagonist pair involved in the regulation of many IFN- γ -inducible genes (35–38). Interestingly, IFN- γ markedly suppressed BH mRNA expression (Fig. 3, A and B). Knock-down and site-directed mutagenesis analyses confirmed that the IRF-1/2-binding site was responsible for the IFN- γ -mediated suppression of BH expression (Fig. 3, B and C). These results clearly show that IRF-1/2 is a mediator of IFN- γ mediated down-regulation of the BH gene in human keratinocytes. On the other hand, Th2 cytokines, IL-4 and IL-13, did not show any direct effect during incubation for 24 h (Fig. 3A). However, these Th2 cytokines significantly suppressed expression of the activator molecules, MZF-1 and Sp-1. Thus, it is plausible that Th2 cytokines negatively regulate BH expression.

We also showed that BH was dramatically down-regulated in lesional and nonlesional AD skins (Fig. 6). This was confirmed with immunohistochemistry, BH enzyme activity, and Western

blot analysis. Although filaggrin mutation is the major risk factor for barrier impairment-related diseases such as AD, mutation analyses indicate that it is still accounts for less than 50% of the incidence in Ireland (5, 39) and only ~20% in Japan (40). We postulated that not only defective filaggrin synthesis but also impaired degradation of filaggrin are involved in the disruption of the barrier function. It is clear that a decrease of NMF results in dry skin, which progresses to barrier disruption. AD is well known to be a Th2-polarized disease. However, recent reports suggest that Th1 cytokines also play a role in AD. For example, intrinsic AD is immunologically characterized by lower expression of IL-4, IL-5, and IL-13 and higher expression of IFN- γ (41). In addition, a Th1 to Th2 shift occurs during the acute phase to chronic phase in AD skin (42). Our results indicate that IFN- γ may play a more important role in AD than has been believed.

In conclusion, our results indicate that BH transcription in human epidermis is regulated in a dual manner. One pathway is under the control of keratinocyte terminal differentiation, and the other is dependent on Th1 and Th2 cytokines. These pathways are mutually related, and it seems likely that the balance would shift easily toward down-regulation of BH expression. Loss of BH results in deficiency of NMF, which in turn leads to dry skin or further to barrier disruption. These results offer new insight into BH regulation and the pathogenesis of AD.

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Supplemental Table S1

Name	Sequence
BH-1216 KpnI (forward)	5´-CCG <u>GGTACCA</u> TCAGAGTTCCTTAGAA-3´
BH-1016 KpnI (forward)	5´-CCG <u>GGTACCA</u> AGGTTTTTACAATCT-3´
BH-816 KpnI (forward)	5´-CAC <u>GGTACCT</u> GGGTAGTGTTCCTTGAA-3´
BH-616 KpnI (forward)	5´-CGA <u>GGTACCT</u> CCTTGTGACATATCGA-3´
BH-444 KpnI (forward)	5´-AAT <u>GGTACCT</u> TGGAGCGGGCCTGA-3´
BH-216 KpnI (forward)	5´-AAT <u>GGTACCA</u> AGGGGGGAGTTTTGTCC-3´
BH-171 KpnI (forward)	5´-AATGA <u>AGGTACCT</u> CAGCCTCCCCGCCG-3´
BH-134 KpnI (forward)	5´-ACG <u>GGTACCA</u> AGCCGTTTTCTTTTC-3´
BH-105 KpnI (forward)	5´-AAT <u>GGTACCT</u> TGCGAGAGACAGGTCG-3´
BH+1 MluI (reverse)	5´-TAAAT <u>ACGCGT</u> TGGCGCCACGCTGCCG-3´

Supplemental Table S2

Gene	Sequence	Annealing
BH	(forward) 5'-TGTGGTTTGGCTGTGATGTT-3'	55°C
	(reverse) 5'-GCACCATCCTGATCATCCTT-3'	
Calpain-I	(forward) 5'-ACATGGAGGCCATCACTTTC-3'	55°C
	(reverse) 5'-GGTCCACGTTGTTCCACTCT-3'	
Sp-1	(forward) 5'-AGCGACCAAGATCACTCCAT-3'	58°C
	(reverse) 5'-TGGGTGACTCAATTCTGCTG-3'	
MZF-1	(forward) 5'-TAGAGCCCTTGCTCACGTTT-3'	58°C
	(reverse) 5'-GGGCATTGTCTAGGTGGAAA-3'	
IRF-1*	(forward) 5'-GAACTCCCTGCCAGATATCGAG-3'	58°C
	(reverse) 5'-TGCTCTTAGCATCTCGGCTGGA-3'	
IRF-2*	(forward) 5'-TGGATGCATGCGGCTAGA-3'	58°C
	(reverse) 5'-CATCTGAAATTCGCCTTCC-3'	
GATA-1	(forward) 5'-ATTGTCAGTAAACGGGCAGG-3'	58°C
	(reverse) 5'-TCTGAATACCATCCTTCCGC-3'	

* Primer reference (38)

Supplemental Table S3

Probe	Sequence
MZF-1	(sense) 5'-CGCGAGGGGGGAGTTCGAGGGGGGAGTTT-3'
	(anti-sense) 5'-GCGCTCCCCCCTCAAGCTCCCCCCTCAAA-3'
Δ MZF-1	(sense) 5'-CGCGAAATGGGAGTTCGAAATGGGAGTTT-3'
	(anti-sense) 5'-GCGCTTTACCCTCAAGCTTTACCCTCAAA-3'
Sp-1	(sense) 5'-TCTCCCAGCCTCAGTCTCCCAGCCTCAG-3'
	(anti-sense) 5'-AGAGGGTCGGAGTCAGAGGGTCGGAGTC-3'
Δ Sp-1	(sense) 5'-TCTCTTGGCCTCAGTCTCTTGGCCTCAG-3'
	(anti-sense) 5'-AGAGAACCGGAGTCAGAGAACCGGAGTC-3'
IRF-1/2	(sense) 5'-CCGGTTTCCTTTTTTCGCGGTTTCCTTTTTTC-3'
	(anti-sense) 5'-GGCCAAAGGAAAAAGCGCCAAAGGAAAAAG-3'
Δ IRF-1/2	(sense) 5'-CCGGTTTCGCGGTTTCGCGGTTTCGCGGTTTC-3'
	(anti-sense) 5'-GGCCAAAGGCGCAAGCGCCAAAGGCGCAAG-3'
GATA-1	(sense) 5'-GCAGCGCAATCCCGGCAGCGCAATCCCGGC-3'
	(anti-sense) 5'-CGTCGCGTTAGGGCCGTCGCGTTAGGGCCG-3'
Δ GATA-1	(sense) 5'-GCAGCGCAAGTACGGCAGCGCAAGTACGGC-3'
	(anti-sense) 5'-CGTCGCGTTCATGCCGTCGCGTTCATGCCG-3'

Supplemental Table S4

	Sequence	Homology (%)
MZF-1	(Consensus) NGNGGGGA (BH-209/-216) AGGGGGGA	96%
Sp-1	(Consensus) A/TCCCA/GGCCA/GC (BH-171/-181) TCCCAGCCTC	89%
IRF-1/2	(Consensus) GGTTTCC/TCTTTTC/G (BH-119/-130) GGTTTCCTTTTC	89%
GATA-1/2	(Consensus) C/GNNGATNNNN (BH-56/-65) CGCAATCCCG	92%