Transcriptional Regulation of *ATP2C1* Gene by Sp1 and YY1 and Reduced Function of its Promoter in Hailey–Hailey Disease Keratinocytes

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Hailey–Hailey disease (HHD) is a blistering skin disease caused by malfunction of the Ca^{2+}-dependent ATPase, ATP2C1. In this study, key regulatory regions necessary for the expression of the gene encoding human *ATP2C1* were investigated. The transient reporter assay demonstrated that region +21/+57 was necessary for activation of the ATP2C1 promoter, and the electrophoretic mobility shift assay demonstrated that the region was recognized by the transcription factors, Sp1 and YY1. In accordance with this result, when Sp1 or YY1 was overexpressed in keratinocytes, an obvious increase in ATP2C1 promoter activity was observed, which was in contrast with the case where a mutant promoter lacking the binding sites for Sp1 and YY1 was used as the reporter. Ca^{2+}-stimulation signal increased nuclear Sp1 proteins and ATP2C1 mRNA levels in normal keratinocytes. In contrast, both these increases were suppressed in keratinocytes from HHD patients. These results indicate that Sp1 and YY1 transactivate the human ATP2C1 promoter via *cis*-enhancing elements and that incomplete upregulation of ATP2C1 transcription contributes to the keratinocyte-specific pathogenesis of HHD. This is a report describing the regulation of the expression of ATP2C1.

Key words: ATP2C1/Hailey–Hailey disease/Sp1/transcription factor/YY1
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Hailey–Hailey disease (HHD) is an autosomal dominant blistering skin disease that is histologically characterized by defects in cell-to-cell adhesion in the suprabasal layers of the epidermis (acantholysis). HHD results from mutations in a Ca^{2+} ATPase, *ATP2C1* (Hu *et al*, 2000; Sudbrak *et al*, 2000). ATP2C1 is a Ca^{2+} ATPase localized to the Golgi apparatus and pumps Ca^{2+} from the cytosol to the Golgi apparatus. In normal keratinocytes, elevated extracellular Ca^{2+} concentration causes differentiation of the cells and induction of differentiated keratinocyte-specific gene expressions. In HHD keratinocytes, however, the cytoplasmic Ca^{2+} response to stimulation caused by increase of extracellular Ca^{2+} concentration is suppressed because of a decrease in ATP2C1 activity. These data suggest that decreased function of ATP2C1 in HHD keratinocytes may account for the suppression of differentiated keratinocyte-specific gene expression that is essential for cell-to-cell adhesion. Recently, Behne *et al* (2003) reported that the decreased expression of ATP2C1 protein in keratinocytes was a probable cause of HHD. This observation suggests that specific control of ATP2C1 expression would give us useful information to restore the decreased function of ATP2C1 in HHD keratinocytes.

Abbreviations: EMSA, electrophoretic mobility shift assay; HHD, Hailey–Hailey disease; NHK, normal human keratinocyte; 5′-RACE, 5′-rapid amplification of cDNA end; 5′-UT, 5′-untranslated

Although ATP2C1 is a critical molecule for HHD, there has been no report on the mechanism for transcriptional regulation of *ATP2C1* gene. In this report, we describe the analysis of transcriptional regulatory elements for the human *ATP2C1* gene expression by using the luciferase reporter assay and electrophoretic mobility shift assay (EMSA), and demonstrate that transcription factors Sp1 and YY1 transactivate *ATP2C1* gene expression by recognizing the *cis*-enhancing elements in 5′-untranslated (5′-UT) region in keratinocyte cells. We also found that Ca^{2+}-induced upregulation of both Sp1 protein level in the nucleus and ATP2C1 transcription were markedly suppressed in HHD keratinocytes.

Results

Determination of transcription start site of the human *ATP2C1* gene To determine the transcription start site of the human *ATP2C1* gene, we performed 5′-rapid amplification of cDNA end (5′-RACE) using total RNA from ATP2C1-positive keratinocyte cell line HaCaT, and found that DNA fragments showing four distinct bands in agarose gel electrophoresis were amplified (Fig 1*A*). The nucleotide sequence of each DNA fragment, which was subcloned onto a plasmid vector, was determined by sequencing analysis. A band of approximately 200 bp contained the sequence corresponding to the 5′-flanking region of *ATP2C1* gene, whereas the other three bands (shown with asterisks in

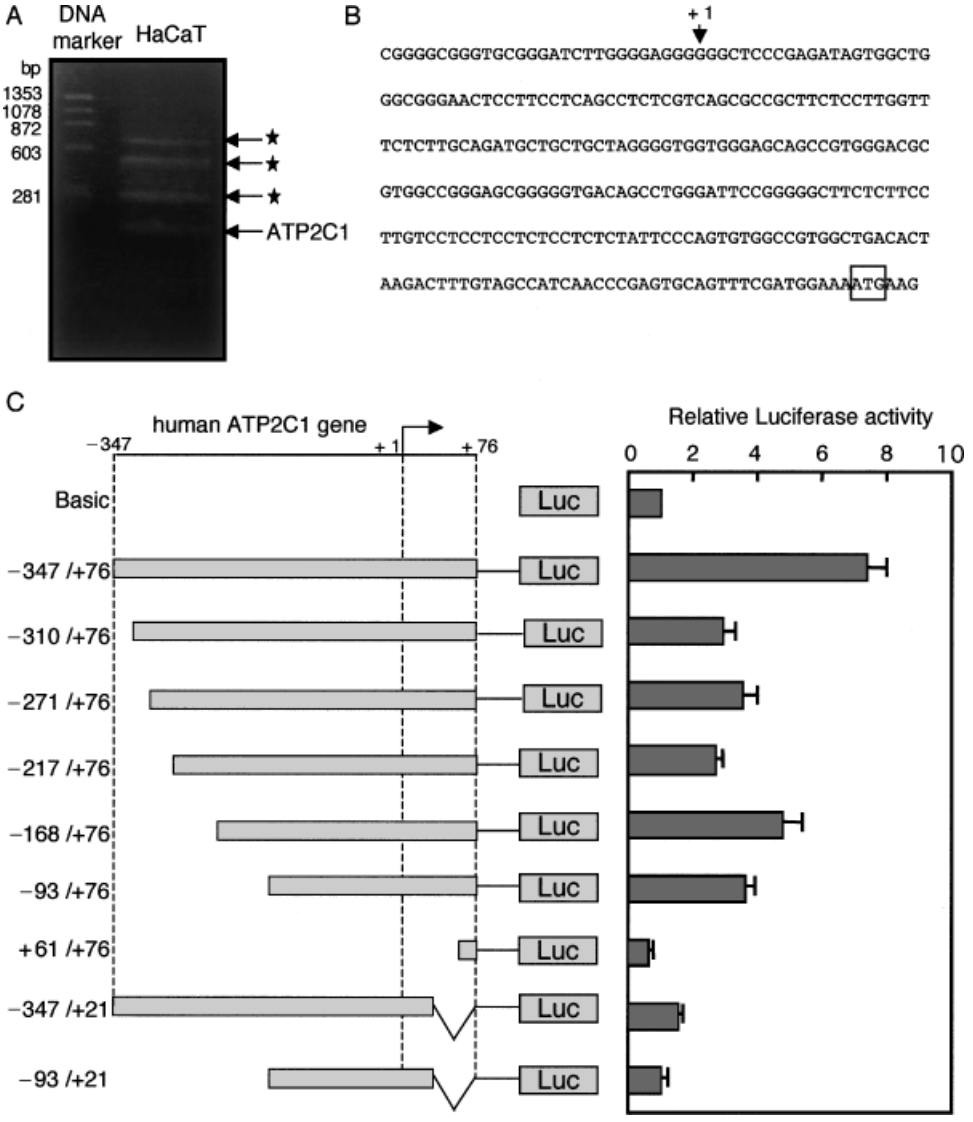

Figure 1
Determination of the transcriptional regulatory region of the human ATP2C1 promoter. (*A*) Agarose gel electrophoresis of 5′-rapid amplification of cDNA end (5′-RACE) products. Bands shown with an asterisk are PCR products of non-related genes. (*B*) 5′-end of ATP2C1 mRNA determined by 5′-RACE analysis. (*C*) Activity of the human ATP2C1 promoter determined by luciferase assay. Nucleotide numbers are shown where the transcriptional start site determined by 5′-RACE is expressed as +1. Deletion constructs of the human ATP2C1 promoter were transiently introduced into HaCaT cells. The luciferase (Luc) activity is given relative to that of pGL3-Bacis (promoterless plasmid), and the results are expressed as the mean ± SE of more than three independent experiments.

Fig 1*A*) were identified to be DNA fragments of non-related genes (data not shown). The transcription start site of the human *ATP2C1* gene was determined (Fig 1*B*) according to the 5′-terminus of each clone.

Analysis of the transcriptional regulatory region of the *ATP2C1* gene To identify the regulatory elements required for the expression of the human *ATP2C1* gene, a series of deletion constructs were generated in which various lengths of the 5′-upstream region of the *ATP2C1* gene were connected to the upstream portion of the luciferase gene. Luciferase activity was then measured in HaCaT cells that were transfected with these reporter plasmids. The longest region (−347/+76) showed apparent promoter activity in comparison with the basic control (Fig 1*C*). But a 37 bp deletion of its 5′-terminus to yield −310/+76 caused a marked decrease in promoter activity, whereas a 217 bp deletion (see −93/+76) did not cause a significant decrease in the activity. These results suggest that the region from −347 to −311 contains *cis*-enhancing element(s). Furthermore, deletion of the region from +22 to +76 resulted in the marked reduction of the promoter activity to levels nearly equal to that of the promoter-less construct. These observations indicate that the positive *cis*-acting element(s) critical for the promoter activity is/are present in the 5′-UT region of the human *ATP2C1* gene.

The above-mentioned deletion analysis suggested that there were two enhancing regions (−347/−311 and +22/+76) involved in the expression of the *ATP2C1* gene. In this study, we analyzed the function of the latter region for the expression of the *ATP2C1* gene. Analysis of the upstream region possibly containing elements to enhance the promoter activity will be performed in the near future and will be described elsewhere. We generated a series of reporter plasmids carrying the *ATP2C1* gene in which nucleotide substitution of 4–5 bp was introduced into +21/+64 of pGL-ATP2C1 (−347/+76) (Fig 2). The mutated promoters, M2–7, showed drastically decreased activity, compared with other promoters. This indicates that the elements essential for the promoter function are present in the region from +21 to +57.

Sp1 and YY1 bind to *cis*-enhancing elements of the *ATP2C1* gene EMSA was performed using nuclear extracts from HaCaT cells, to identify nuclear proteins binding to the above-mentioned essential elements of 5′-UT. When a 5′-FITC-labeled double-stranded oligonucleotide of +21/+44

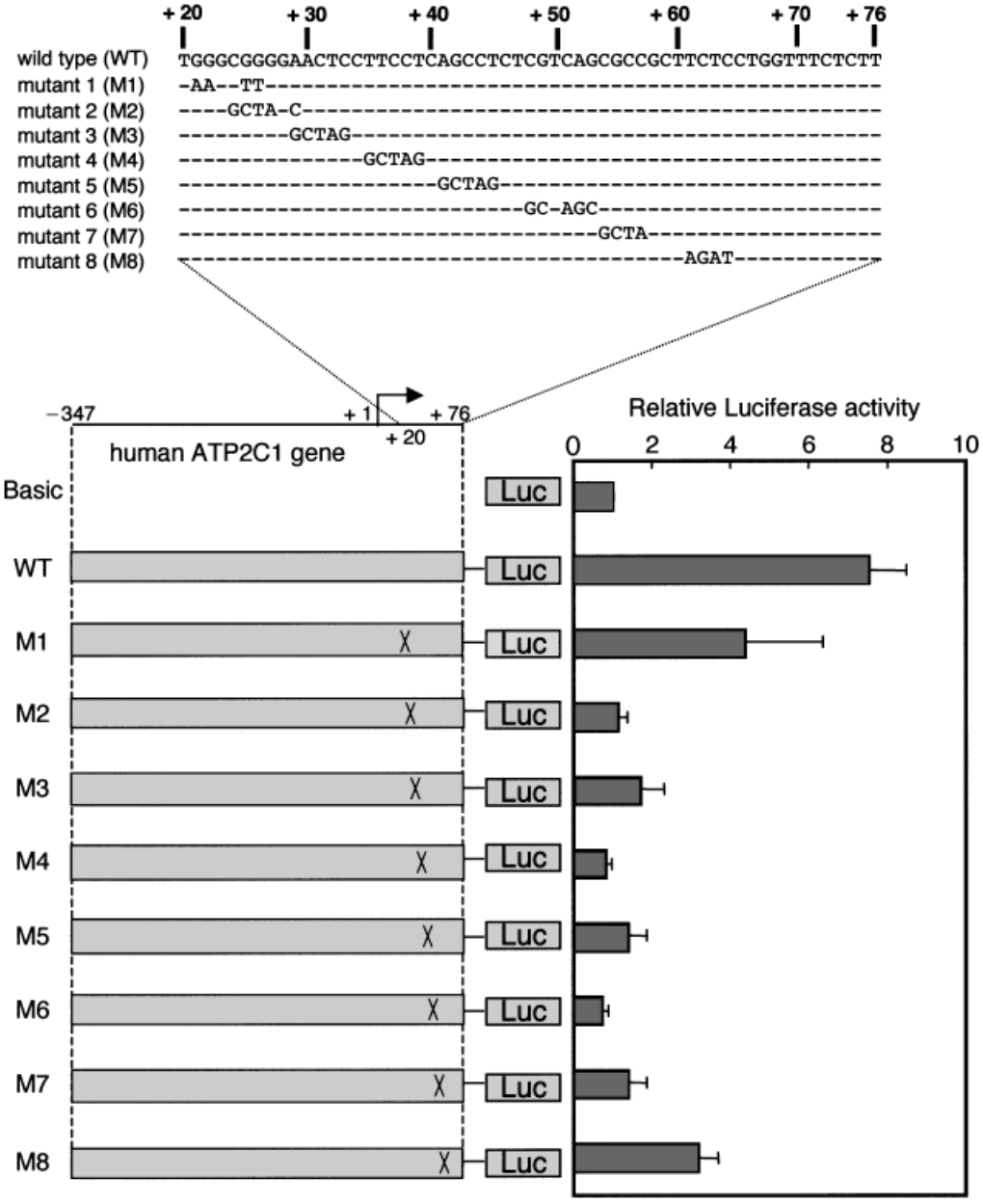

Figure 2
Determination of *cis*-enhancing elements in the 5′-untranslated region of the human ATP2C1 gene by site-directed mutagenesis. Mutations were introduced into +21/+64 of the reporter plasmid carrying the human ATP2C1 promoter (−347/+76). Various mutant plasmids were introduced into HaCaT cells. Nucleotides that differed from the original sequences are shown, and lines represent unchanged nucleotides in mutant plasmids. Results are expressed as the mean ± SE of more than three independent experiments. WT, wild type; M1–8, mutant 1–8.

(probe 1) was used as a probe, several shift bands were observed (Fig 3*A*). Motif analysis using the program, TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), revealed the presence of recognition motifs for various transcription factors, including Sp1, YY1, NF-κB, and the Ets family, in this region. To confirm the binding of these transcription factors to +21/+44 of the *ATP2C1* gene, EMSA was performed in the presence of anti-Sp1, -YY1, -NF-κB, -Elf-1, or -Elk-1 antibody (Fig 3*A*). When anti-Sp1 antibody was added in a protein–DNA mixture, the most slowly migrating band was diminished but a super shift band appeared (Fig 3*A*). EMSA in the presence of anti-YY1 antibody also resulted in the disappearance of a shift band (Fig 3*A*, *lane 4*), whereas addition of other antibodies did not affect the EMSA profile. These results indicate that Sp1 and YY1 bind to +21/+44 of the *ATP2C1* gene. To confirm that Sp1 and YY1 bound to this region, EMSA was performed with recombinant Sp1 and YY1 prepared by an *in vitro* transcription/translation system. As shown in Fig 3*B*, the specific shift band showing mobility identical to that seen with the nuclear protein was generated when recombinant Sp1 was used. Furthermore, disappearance of this specific band and simultaneous appearance of a supershift band were observed in the presence of anti-Sp1 antibody as seen in the case in which nuclear proteins were used. When an *in vitro* transcription/translation reaction mixture including YY1 cDNA as a template was added, a specific shift band showing mobility identical to that of the band seen in EMSA using nuclear extract appeared (Fig 3*C*). Furthermore, this band disappeared in the presence of anti-YY1 antibody (Fig 3*C*).

For further identification of the recognition sequence of Sp1, we performed competition assays by using 50- or 100-fold excess amounts of unlabeled wild-type or mutant-type double-stranded oligonucleotide. The most slowly migrating band disappeared when the mixture was incubated in the presence of WT, competitor 1, 5, or 6 (Fig 4*B*). In contrast, addition of competitor 2, 3, or 4 was less effective when used to compete with the labeled probe (Fig 4*B*). These

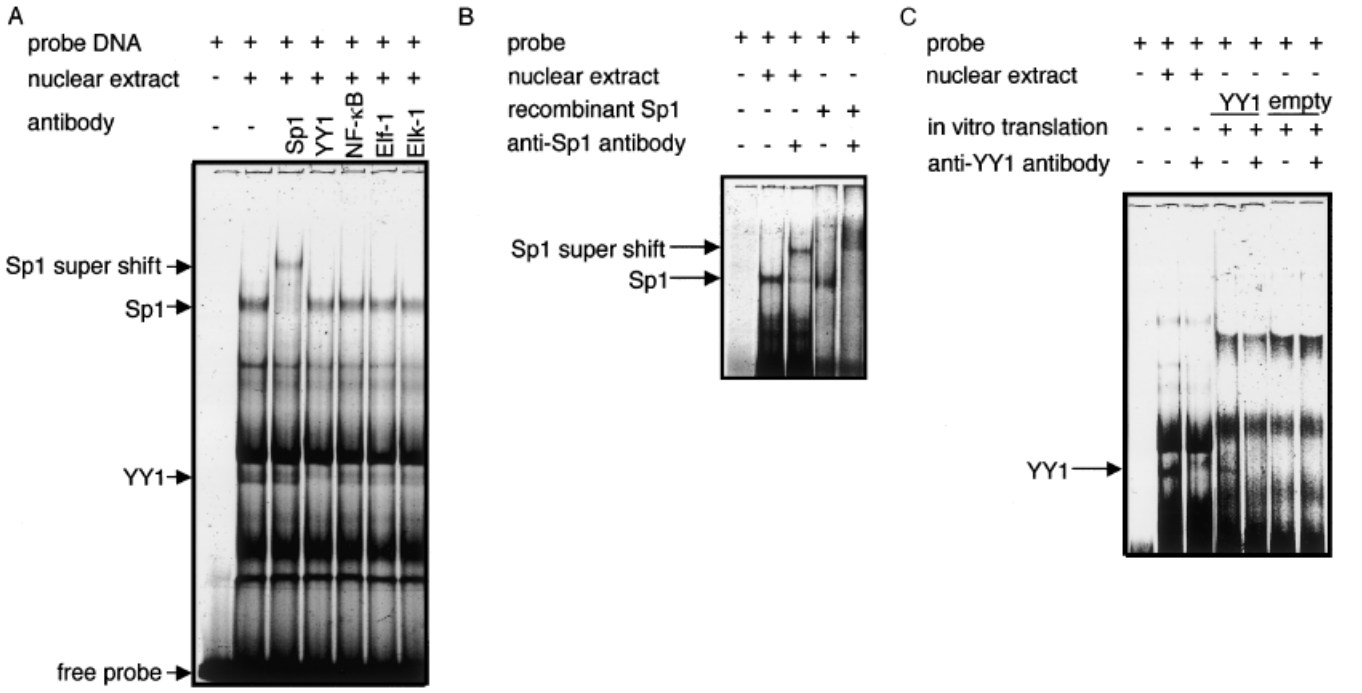

Figure 3
Identification of nuclear proteins binding to the 5′-untranslated region of the human ATP2C1 gene by electrophoretic mobility shift assay (EMSA). (*A*) EMSA was performed with FITC-labeled probe 1 and nuclear extract from HaCaT cells. *Lane 1*, probe only; *lanes 2–7*, probe with nuclear extract. Added antibody is shown above each lane. (*B*) Mobility profile of recombinant Sp1. *Lanes 2* and *3*, with nuclear extract; *lanes 4* and *5*, with recombinant Sp1; *lanes 1*, *2*, and *4*, without anti-Sp1 antibody; *lanes 3* and *5*, with anti-Sp1 antibody. (*C*) Mobility profile of *in vitro*-translated YY1. *Lanes 2* and *3*, with nuclear extract; *lanes 4* and *5*, with *in vitro* translated YY1; *lanes 6* and *7*, with *in vitro* transcription/translation mixture without template cDNA; *lanes 1*, *2*, *4*, and *6*, without anti-YY1 antibody; *lanes 3*, *5*, and *7*, with anti-YY1 antibody.

results indicate that GGGGAACTCCTT (+25/+36) serves as a core sequence for the binding of Sp1. Similarly, the YY1-binding site was identified as CCTTCCTCAGCC (+37/+44; data not shown).

Sp1 and YY1 transactivate the ATP2C1 promoter To elucidate the transactivating role of Sp1 and YY1 in ATP2C1 promoter function, the effect of overexpression of Sp1 or YY1 on the ATP2C1 promoter activity was assessed by co-expression study in normal human keratinocyte (NHK) cells. When the NHK cells were co-transfected with Sp1- or YY1-expression plasmid, a significantly higher amount of Sp1 or YY1 was overproduced in these cells (Fig 5*A*). The ATP2C1 promoter (−347/+76) was upregulated 3- and 2-fold by exogenously expressed Sp1 and YY1, respectively (Fig 5*B*). In contrast, exogenous Sp1 and YY1 produced no significant effect when the mutant promoter lacking recognition motifs of Sp1 and YY1 (−347/+21) or when basic control was used (Fig 5*B*). These results suggest that Sp1 and YY1 transactivate the ATP2C1 promoter via binding motifs in 5′-UT in keratinocytes.

Effect of Ca^{2+} on transcription level of *ATP2C1* and nuclear accumulation of Sp1 Ca^{2+} stimulation induces nuclear localization of Sp1 and a terminal differentiation program of keratinocytes similar to that observed in the upper epidermal layers, including induction of differentiation-related genes (Santini *et al*, 2001). Therefore, we analyzed the effect of Ca^{2+}-stimulation on *ATP2C1* transcription and the amount of nuclear Sp1 and YY1. To examine transcription levels of *ATP2C1* in keratinocytes, we performed a semi-quantitative PCR using total RNA prepared from undifferentiated (cultured in 0.06 mM Ca^{2+}) NHK and differentiated (cultured in 1.2 mM Ca^{2+}) NHK, and found that mRNA for ATP2C1 was markedly increased (Fig 6*B*). As expected, Ca^{2+} stimulation induced the accumulation of Sp1 proteins in the nucleus (Fig 6*A*). On the other hand, Ca^{2+} stimulation was Sp1 specific, because the amount of nuclear YY1 was not affected by the stimulation (Fig 6*A*). These results suggest that Ca^{2+} stimulation induces the accumulation of Sp1 in the nucleus, and the accumulated transcription factor promotes the transcription of *ATP2C1*.

Nuclear Sp1 level and *ATP2C1* transcription level in keratinocytes from HHD patients HHD keratinocytes show a decrease in cytoplasmic Ca^{2+} response to stimulation with raised extracellular Ca^{2+} because of the decreased function of ATP2C1 that controls Golgi Ca^{2+} stores (Hu *et al*, 2000; Behne *et al*, 2003). This suggested the possibility that transcription of genes that should be upregulated in differentiated keratinocytes might be abnormal in HHD keratinocytes. We hypothesized that *ATP2C1* transcript may be decreased in differentiated keratinocytes from HHD patients. To explore this possibility, we compared nuclear Sp1 proteins levels and *ATP2C1* transcription levels between undifferentiated and differentiated keratinocytes from HHD patients and controls. When relative Sp1 protein levels were calculated based on the intensity of bands detected in western blotting with nuclear extract by using an irrelevant USF2 protein, in which the amount in the nucleus is not affected by Ca^{2+} stimulation, as a control (Fig 7*A*), Sp1 proteins in the nucleus of control keratinocytes were upregulated in response to Ca^{2+} stimulation as well as that

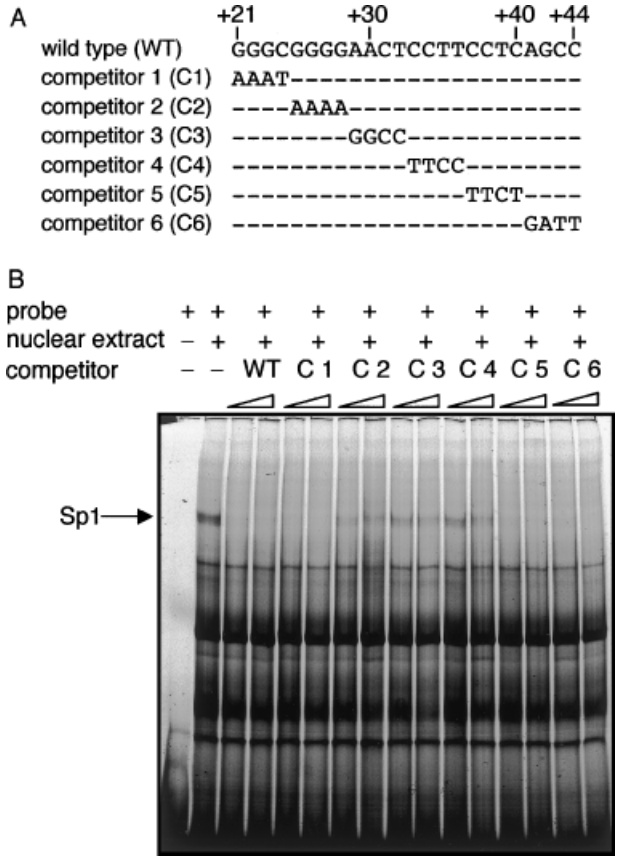

Figure 4
Determination of binding sites of transcription factor Sp1. *Lane 1*, probe only; *lanes 2–16*, with nuclear extract; *lanes 3–4*, *5–6*, *7–8*, *9–10*, *11–12*, *13–14*, and *15–16*, with competitor WT, C1, C2, C3, C4, C5, and C6, respectively. The amount of competitor was 50-fold excess in lanes 3, 5, 7, 9, 11, 13, and 15, and 100-fold excess in lanes 4, 6, 8, 10, 12, 14, and 16.

of NHK, whereas this upregulation was suppressed in keratinocytes from HHD patients (Fig 7*B*). When ATP2C1 mRNA levels were analyzed by real-time PCR, ATP2C1 mRNA levels were dramatically increased in differentiated control keratinocytes and NHK. Ca^{2+} stimulation did not affect, however, ATP2C1 mRNA levels in keratinocytes from HHD patients (Fig 7*C*). These results demonstrate that expression of ATP2C1 mRNA was upregulated in normal differentiated keratinocytes but not in differentiated HHD keratinocytes.

Discussion

The human *ATP2C1* gene has been identified to be an HHD-related gene, because the mutation of *ATP2C1* causes this disease (Hu *et al*, 2000; Sudbrak *et al*, 2000). Recently, it was reported that protein levels of ATP2C1 were decreased in keratinocytes from HHD patients and that abnormal Ca^{2+} signaling was seen in HHD keratinocytes (Behne *et al*, 2003). Therefore, information on the mechanism of gene expression might be useful for further understanding the cause of HHD and for development of a new therapeutic method for the disease. There has been no report, however, analyzing the mechanism for *ATP2C1* gene expression. In this study, we analyzed the promoter structure of the human *ATP2C1* gene and found the 5′-UT region containing the critical *cis*-enhancing elements by using the transient reporter assay. In addition, transcription factors Sp1 and YY1 were identified to bind to the elements by EMSA. We also showed that these transcription factors transactivated the ATP2C1 promoter via the *cis*-enhancing elements in keratinocyte cells. Furthermore, we found that upregulation of *ATP2C1* transcription caused by Ca^{2+}-stimulation signal was markedly suppressed in HHD keratinocytes, probably because of reduced upregulation of nuclear Sp1 protein.

The *ATP2C1* gene is transcribed at a high level in human epidermal keratinocytes, although transcription of *ATP2C1* was also observed in other human tissues at moderate levels (Hu *et al*, 2000). Both Sp1 and YY1 are expressed in various cell types. Therefore, it would be reasonable to think that these transcription factors might be involved in the expression of ATP2C1 in a cell-type-independent manner. In other instances, however, Sp1 and YY1 have been reported to be involved in the transcriptional regulation of the keratinocyte-specific genes related to terminal differentiation of keratinocytes, such as loricrin (Jang and Steinert, 2002), transglutaminase 3 (Lee *et al*, 1996), and involucrin (Lopez-Bayghen *et al*, 1996). Considering that relatively high levels of Sp1 expression are observed in keratinocytes (Saffer *et al*, 1991), keratinocyte-specific gene expression mediated by Sp1 may be controlled by the expression level of Sp1. It is also possible to postulate that another keratinocyte-specific transcription factor or cofactor might be involved in the expression of keratinocyte-specific genes in a cooperative manner with Sp1 and YY1.

By EMSA with competitive oligonucleotides, Sp1 was identified to bind to the region $+25/+36$ (Fig 4). In the region that bound to Sp1, no complete canonical Sp1-binding sequence, 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ (Thiesen and Bach, 1990; Nagaoka *et al*, 2002), was found. We assumed firstly that a palindromic sequence, GGAACTCC ($+27/+34$), which is similar to the 3′-half of the Sp1-consensus sequence, could serve as the Sp1-binding site in the human *ATP2C1* gene. When we performed co-expression analysis using a mutant reporter plasmid with nuclear replacements at $+29/+33$ (M3 in Fig 2), however, promoter activity was still upregulated by exogenous Sp1 (data not shown). This result suggests the presence of other Sp1-binding sequences in this region. The TGGGCGGGG ($+20/+28$) sequence is also similar to the optimal consensus-binding sequence of Sp1. Therefore, the sequence may be an alternative candidate for the Sp1-binding site. As for the YY1-binding site, the CAG sequence of $+40/+42$ might serve as a core motif, considering that NC(A/C)(T/G)NNN was shown to be recognizable by YY1 (Shrivastava and Calame, 1994). Luciferase activity directed by a mutant reporter plasmid with replacement at $+41/+45$, however, was increased by co-expression of YY1. Considering that the YY1-binding sequence is relatively flexible, the complicated result may be explained by the presence of other YY1-bindable sequences in this area. Alternatively, the close proximity of Sp1- and YY1-binding sites might make the effect of each transcription factor more complex. Further detailed analysis will be required to reveal the cooperative mechanism between Sp1 and YY1 and to clarify the involvement of YY1. Nonetheless, this study suggests that Sp1 and YY1 transactivated the human

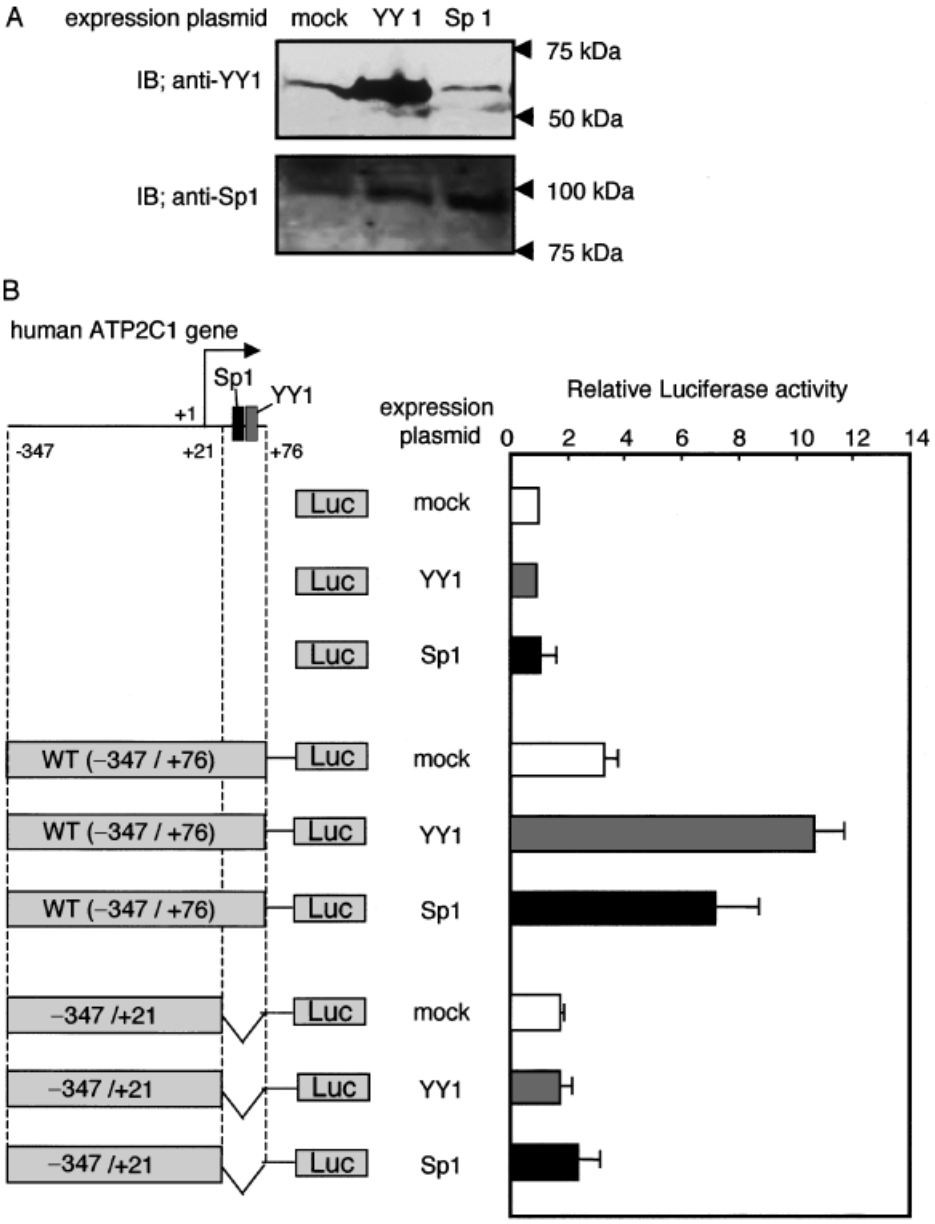

Figure 5
Transcription activation of the ATP2C1 promoter by Sp1 and YY1. (*A*) Western blotting analysis of transfectants. To examine protein levels of transcription factors, 1/5 volumes of whole transfected cell lysate in each well of six-well plate were subjected to western blot analysis. (*B*) Two micrograms of reporter plasmids were introduced with 100 ng pCR3.1 (mock), pCR-Sp1, or pCR-YY1 into normal human keratinocytes. The relative promoter activity is represented as the ratio to the luciferase activity in the cells transfected with pGL3-Basic and mock vector. Typical results obtained with triplicate samples are shown and similar results were obtained with other independent experiments.

ATP2C1 promoter via binding sequences located between $+22/+76$.

As shown in Fig 1*C*, deletion of the $-347/-311$ region caused an apparent decrease in the transcription activity of the promoter. Further, nucleotide replacement in 5′-UT caused a marked decrease in the promoter activity (Fig 2), and mutated promoter M8 showed slightly decreased activity in comparison with that of the wild type one. These results suggest that other transcription factors or cofactors might be involved in the regulation of *ATP2C1* gene expression. With regard to the latter region, when we performed EMSA by using $+41/+60$ as a probe (probe 2), did not detect a specific band shift (data not shown). Additional detailed analysis of these regions would be of benefit in further clarifying these regulatory mechanisms.

The region within the 5′-UT was essential for the promoter function of the human *ATP2C1*. Although a regulatory element for the gene expression is often found within its own promoter or upstream of the promoter, there are increasing number of instances in which the 5′-UT contains a regulatory element (Yamamoto *et al*, 1999; Morii *et al*, 2001; Akizawa *et al*, 2003). Sp1 also binds to the 5′-UT of the insulin-like growth factor I receptor gene (Beitner-Johnson *et al*, 1995). Further study on the ATP2C1 promoter may be of benefit to characterize the functional and structural differences between the typical promoter and the 5′-UT-dependent promoter.

It is known that ATP2C1 function is decreased in HHD keratinocytes. In this study, we have shown that upregulation of ATP2C1 expression, which is induced by Ca^{2+}-stimulation signal for cell differentiation in normal keratinocytes, was not observed in HHD keratinocytes. Therefore, it is reasonable to think that the difference in ATP2C1 function may become larger after differentiation between normal keratinocytes and HHD keratinocytes. We may assume that it is a possible reason why HHD is usually found in the upper layer of the epidermis where keratinocytes are in the final stage of differentiation. In previous reports, low protein levels were observed in ATP2C1 mutants, which were overproduced in COS-1 cells by transiently introduced

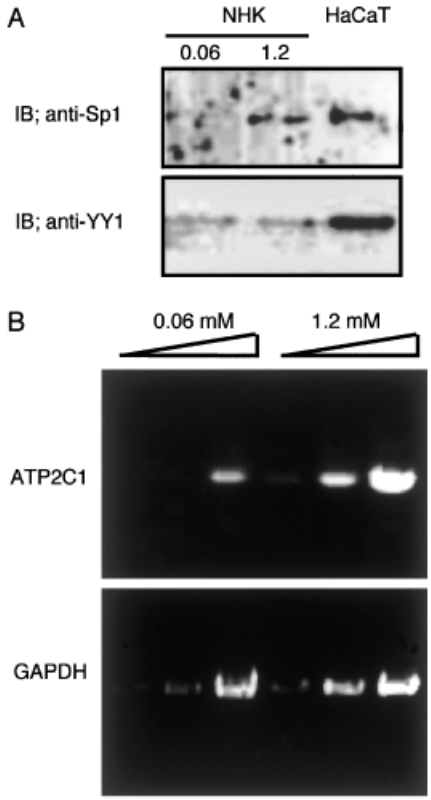

Figure 6
Effect of Ca^{2+}-stimulation signal on the amount of nuclear transcription factors and transcription level of ATP2C1 in normal human keratinocyte (NHK). (*A*) The amount of Sp1 and YY1 proteins in nucleus of NHK and HaCaT cells. Five micrograms of nuclear extract was applied onto each lane and analyzed by western blotting. *Lane 1*, nuclear extract from NHK cells cultured in the presence of 0.06 mM Ca^{2+}; *lane 2*, nuclear extract from NHK cells stimulated with 1.2 mM Ca^{2+} for 24 h; *lane 3*, nuclear extract from HaCaT cells. (*B*) Semiquantitative PCR to analyze the transcription level of ATP2C1. PCR products obtained after 25 (*lanes 1* and *4*), 28 (*lanes 2* and *5*), and 31 (*lanes 3* and *6*) cycles were applied onto agarose gel and detected by ethidium bromide staining. One microgram total RNA from non-stimulated NHK (*lanes 1–3*) and from stimulated NHK (*lanes 4–6*) were used as the template for RT.

plasmids, whereas transcription levels of ATP2C1 mutants were comparable (Fairclough *et al*, 2003, 2004). The reduced protein levels were suggested to be probably because of a rapid rate of mutant protein degradation. Considering this, the stability of mutant proteins might be critical for the expression level of ATP2C1 proteins. Therefore, there is a possibility that the level of ATP2C1 mutant proteins was further decreased in HHD keratinocytes by both the effect of suppressed transcription and enhanced degradation. Since we did not analyze the protein levels of ATP2C1 in this study, further analysis will be required to clarify this possibility. In this study, Ca^{2+} stimulation was used to induce development of keratinocytes. Since Ca^{2+} signaling itself is defective in Hailey–Hailey keratinocytes, there is a possibility that suppression of ATP2C1 promoter in HHD keratinocytes was enhanced by the deficient function of ATP2C1. *In vivo* analysis of transcription and translation level of ATP2C1 in keratinocytes of HHD is required for further clarification of the involvement of ATP2C1 promoter in the pathogenesis of HHD.

Materials and Methods

Cell culture HaCaT cells were cultured in DMEM (Sigma-Aldrich, St Louis, Missouri) supplemented with 10% FBS (Sigma-Aldrich), 100 U per mL penicillin, and 100 μg per mL streptomycin. NHK, derived from normal newborn foreskin, were purchased from KURABO (Osaka, Japan) and maintained in a serum-free standard medium Epilife-KGM (KURABO) containing 0.06 mM Ca^{2+}, 10 μg per mL insulin, 0.1 ng per mL hEGF, 0.5 μg per mL hydrocortisone, 50 μg per mL gentamicin, 50 ng per mL amphotericin-B, and 0.4% BPE. Second-to-fourth-passage keratinocytes that were 60%–90% confluent in a monolayer were used for experiments. We used HaCaT cells in a reporter assay and EMSA to identify transcription factors essential for *ATP2C1* gene regulation after confirmation that HaCaT is an ATP2C1-positive cell line, because cell lines generally express a higher amount of transcription factors compared with primary cells and are used for identification of *cis*-elements and *trans*-activators. NHK were used to evaluate the effect of exogenous transcription factors by co-expression analysis, because the amount of endogenous target transcription factors in NHK was confirmed to be lower than that of HaCaT. NHK were also used to analyze levels of transcription factor proteins in nucleus and ATP2C1 transcription in response to Ca^{2+} stimulation, because NHK is a primary keratinocyte that differentiates in response to an increase of extracellular Ca^{2+} concentration.

Selection of HHD patients and controls HHD individuals were patients of Juntendo University Hospital, who were diagnosed before entry into the study, demonstrating the typical clinical findings and histopathology, i.e., blistering and erosions in characteristic locations, and acantholysis of suprabasal cells without apoptosis. Keratinocytes from HHD patients and healthy control were generated from a skin biopsy of the trunk and were used for experiments in their second-to-fourth passages. HHD patient #1 in this study was described in a previous report (Ikeda *et al*, 2001) as JHHD-10 with an in-frame deletion in *ATP2C1* gene, 2215delATT, causing deletion of Ile at 739th.

The Ethics Committee of Juntendo University School of Medicine approved this study. This study was conducted according to the Declaration of Helsinki Principles and participants gave their written informed consent.

RT-PCR and 5′-RACE Total RNA was prepared from HaCaT cells using TRIzol reagent (Invitrogen, Leek, the Netherlands). A 5′-RACE kit (Invitrogen) was used for 5′-RACE analysis. The following oligonucleotides were used as primers for 5′-RACE: GSP1 (5′-CGAAACTGCACTCGGGTTGATGGCTACAAA-3′), GSP2 (5′-AATAGAGAGGAGAGGAGGAGGACAAGGAG-3′), and GSP3 (5′-CACCCCTAGCAGCAGCATCTGCAAGAGAAA-3′). Each PCR product was subcloned into pCR2.1 by using a TOPO TA cloning kit (Invitrogen), and nucleotide sequences were determined by an ABI PRISM377 DNA sequencer (Applied Biosystems, Foster City, California).

Plasmid construction The 5′-flanking region (approximately 450 bp) including a 5′-UT region of human *ATP2C1* gene was prepared by PCR using 5′-c*agatct*GCCCTCTCTCCCTCCCTTCCT-3′ and 5′-c*aagctt*AAGAGAAACCAGGAGAAGCGGCGCTGACGA-3′ (replaced nucleotides for introduction of *Bgl*II and *Hin*dIII sites (in italics) are shown as small letters) as the primers and the human genomic DNA prepared from peripheral blood by a DNA quick kit (Dainippon Pharmaceutical, Osaka, Japan) as the template. The amplified genomic DNA fragment were inserted into the *Bgl*II–*Hin*dIII site of pGL3-Basic (Promega, Madison, Wisconsin) after digestion with restriction endonucleases, *Bgl*II and *Hind*III (Takara BIO, Otsu, Japan). The nucleotide sequences of inserted DNA fragment was confirmed by sequencing analysis. Other plasmids containing a variety of 5′- or 3′-truncation of the ATP2C1 promoter region connected to the upstream region of the luciferase gene were constructed in a similar manner using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California) and

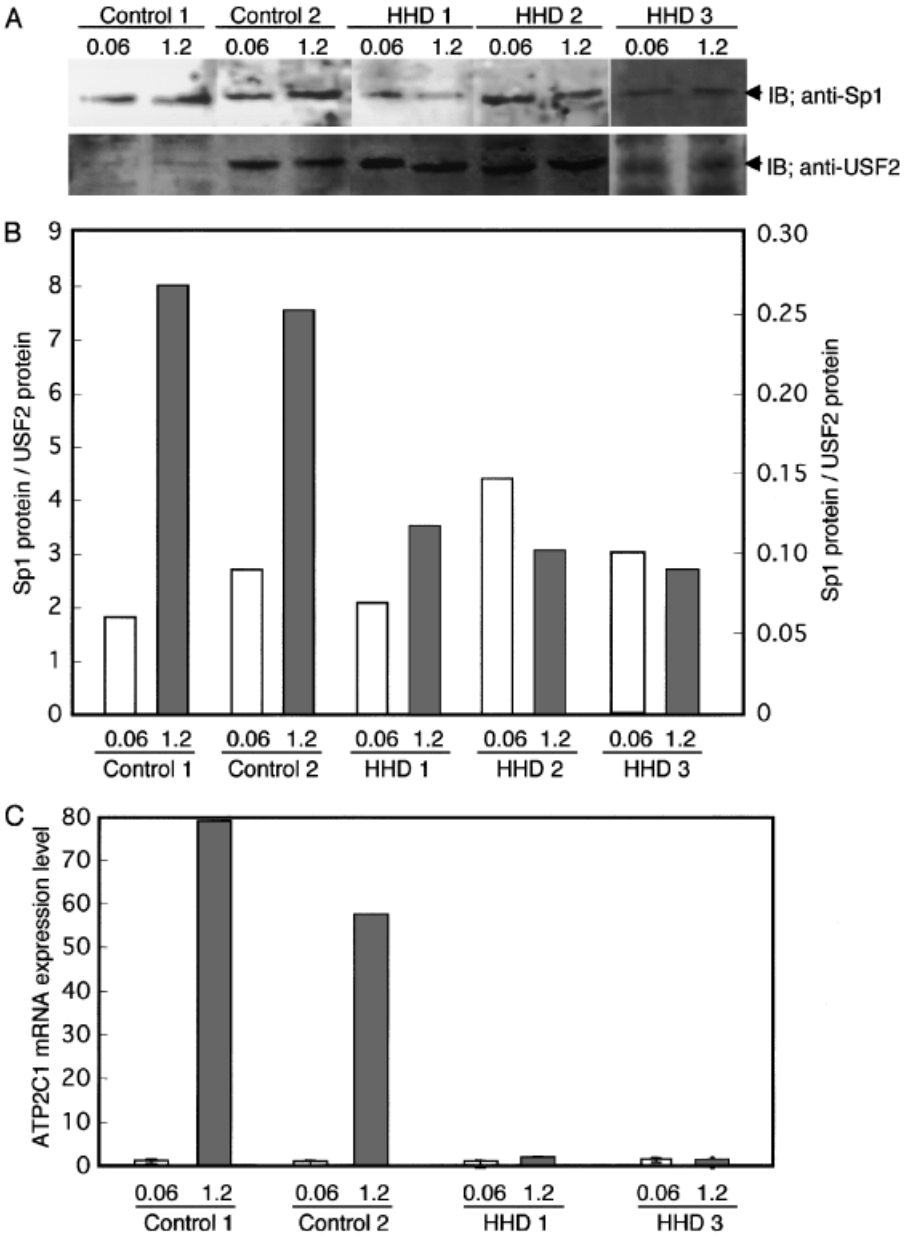

Figure 7
Levels of nuclear Sp1 proteins and ATP2C1 mRNA in keratinocytes from Hailey–Hailey disease (HHD) patients. (*A*) Western blotting analysis of Sp1 (*top*) and USF2 (*bottom*) proteins in the nucleus of keratinocytes from normal and HHD individuals. *Control 1*, commercially obtained normal human keratinocytes; *Control 2*, normal human keratinocytes from volunteer; HHD, keratinocytes from HHD patient. The numbers of "Control" and "HHD" indicate the corresponding individuals in (*A–C*). (*B*) The relative Sp1 protein levels in the nucleus. Densitometric analysis was used to assess the relative Sp1 protein levels normalized to the irrelevant nuclear protein, USF2. The scale of Y-axis on the left is for control 1 and HHD 3, and the scale on the right is for other cells. (*C*) Quantification of ATP2C1 mRNA levels in control and HHD keratinocytes by real-time PCR. The expression level of the ATP2C1 (ATP2C1/GAPDH) of each individual is represented as the ratio to that of non-stimulated normal human keratinocyte.

restriction endonucleases. All of the constructs in which 4–5 bp were replaced with others were produced by site-directed mutagenesis. The expression plasmid, pCR-YY1 (Nishiyama *et al*, 2002, 2003), was used for *in vitro* transcription/translation of YY1 and co-expression analysis. The plasmid pCR-Sp1 for expression of Sp1 in keratinocytes was generated by cloning the coding region of Sp1 cDNA (accession number; BC062539), which was amplified from total RNA prepared from NHK by RT-PCR using 5′-CTGCCACC**ATG**AGCGACCAAGATCACTCCA-3′ (the translational initiation codon is shown in bold) and 5′-CCGGGTGCCTGATC**TCA** GAAGCCATTGCCA-3′ (the termination codon is shown in bold) as primers, into pCR-3.1 (Invitrogen) in the appropriate orientation.

Transfection and luciferase assay HaCaT cells (1×10^5) were transfected with 2 μg reporter plasmid, and 25 ng pRL-CMV (Promega), which was used as the internal control of transfection efficiency, by Fugene6 (Roche Diagnostics, Indianapolis, Indiana) according to the manufacturer's instruction. For co-expression analysis, NHK (1×10^5) were transfected with 2 μg reporter plasmid, and 25 ng pRL-null (Promega) with 100 ng expression plasmid, pCR-3.1 (mock), pCR-Sp1, or pCR-YY1. After 20–24 h of culture, luciferase activity of each whole-cell lysate was measured using a Dual-luciferase assay kit (Promega) and a luminescence detector, Micro Lumat Plus (Berthold, Postfach, Germany) as described previously (Akizawa *et al*, 2003; Maeda *et al*, 2003).

EMSA Probes for EMSA were prepared by annealing 5′-FITC-labeled synthesized oligonucleotides of complementary sequences. The sequences of sense strand of probe were: probe-1, 5′-GGGCGGGGAACTCCTTCCTCAGCC-3′ and probe-2, 5′-AGCCTCTCGTCAGCGCCGCT-3′ (Invitrogen). The non-labeled double-stranded oligonucleotides (see Fig 4*A*) were prepared in a similar manner and used as a competitor. Nuclear extracts from HaCaT cells were prepared as previously described (Nishiyama *et al*, 1999; Maeda *et al*, 2003). Recombinant Sp1 protein was purchased from ProteinOne.com (College Park, Maryland). *In vitro* transcription and translation of YY1 was performed with a TNT T7 Quick coupled transcription/translation system (Promega) using pCR-YY1 as the template. Five picomole probes were mixed with the nuclear extract of 5 μg of protein, 1.2 μg of recombinant Sp1, or 1 μL of *in vitro* transcription/translation mixture containing YY1 protein. This mixture was applied onto a 4% polyacrylamide gel. One microgram of each antibody, anti-Sp1, anti-YY1, anti-NFκB p65, anti-Elf-1, and anti-Elk-1 (Santa Cruz Biotechnology, Santa

Cruz, California) was added into the protein–probe mixture to identify the nuclear proteins binding to the probe–DNA. Competition assay was performed as described previously (Hasegawa *et al*, 2003). Gels were subjected to a fluorescence detector, FluorоImager 595 (Molecular Dynamics, Sunnyvale, California).

Western blotting analysis Whole cell or nuclear extracts prepared by the method described above were subjected to western blotting analysis. The amount of subjected samples is described in each figure legend. Western blotting analysis was performed as described previously (Ito *et al*, 2005). In brief, anti-Sp1, anti-YY1 antibodies that were used in EMSA as described above, and anti-USF2 antibody (Santa Cruz Biotechnology) were used as the primary antibodies. Alexa Fluor 800 goat anti-mouse IgG, Alexa Fluor 680 goat anti-mouse IgG, or Alexa Fluor 680 goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies to detect Sp1, YY1, and USF2, respectively. Infrared fluorescence on membranes was detected by the Odyssey infrared imaging system (model ODY-9201-SC; LI-COR, Lincoln, Nebraska).

Quantification of ATP2C1 mRNA Total RNA was prepared from NHK cells as described above. Reverse transcription was performed using a ThermoScript First-Strand Synthesis System for RT-PCR (Invitrogen). The Advantage 2 polymerase mix (BD Clontech, Palo Alto, California) was used for semi-quantitative PCR with the following primer set: 5′-TGACAGGTGAGACAACGCCTTGTTC-3′, and 5′-TCACTGTGACCACAATGGGGAGAC-3′ to detect ATP2C1 expression, and a primer set to detect GAPDH expression was purchased from BD Clontech. PCR products after an appropriate number of cycles were subjected to agarose gel electrophoresis. The amount of ATP2C1 mRNA was also quantified using a 7500 Real-Time PCR System (Applied Biosystems) with an Assays-on-Demand gene expression product (no. Hs00205122_m1 for human ATP2C1) and TaqMan Universal PCR Master Mix (Applied Biosystems). The expression level of ATP2C1 was normalized to that of GAPDH as described previously (Nishiyama *et al*, 2004). The ATP2C1 expression level in Fig 7*C* is shown as the ratio to that of non-stimulated NHK according to the following equations:

$$\text{The ATP2C1 mRNA expression level} = 2^{(C_t\,\text{sample})-(C_t\,\text{control})}$$

$$C_t\,\text{sample} = (C_t\,\text{value of ATP2C1 of each sample}) - (C_t\,\text{value of GAPDH of each sample})$$

$$C_t\,\text{control} = (C_t\,\text{value of ATP2C1 of non-stimulated NHK}) - (C_t\,\text{value of GAPDH of non-stimulated NHK})$$

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