NF-Y and Sp1/Sp3 are involved in the transcriptional regulation of the peptidylarginine deiminase type III gene (*PADI3*) in human keratinocytes

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Human peptidylarginine deiminase type III gene (*PADI3*) encodes a crucial post-translational modification enzyme that converts protein-bound arginine residues into citrulline residues. Its expression is restricted to a few cell types, including keratinocytes in the granular layer of the epidermis and in the inner root sheath of hair follicles. In these cells, the enzyme is involved in terminal processing of intermediate filament-binding proteins such as filaggrin and trichohyalin. To study the molecular mechanisms that control the expression of *PADI3* in human keratinocytes at the transcriptional level, we characterized its promoter region using human keratinocytes transfected with variously deleted fragments of the 5′-upstream region of *PADI3* coupled to the luciferase gene. We found that as few as 129 bp upstream from the transcription initiation site were sufficient to direct transcription of the reporter gene. Electrophoretic mobility-shift and chromatin immunoprecipitation assays revealed that NF-Y (nuclear factor Y) and Sp1/Sp3 (specificity protein 1/3) bind to this region *in vitro* and *in vivo*. Moreover, mutation of the Sp1- or NF-Y-binding motif markedly reduced *PADI3* promoter activity. Furthermore, Sp1 or NF-YA (NF-Y subunit) small interfering RNAs effectively diminished *PADI3* expression in keratinocytes cultured in both low- and high-calcium medium. These data indicate that *PADI3* expression is driven by Sp1/Sp3 and NF-Y binding to the promoter region.

Key words: CCAAT box, GC box, nuclear factor Y (NF-Y), peptidylarginine deiminase type III gene (*PADI3*), specificity protein 1/3 (Sp1/Sp3), transcriptional regulation.

INTRODUCTION

PAD (peptidylarginine deiminase; EC 3.5.3.15) is a post-translational modification enzyme that catalyses the conversion of protein-bound arginine residues into citrulline residues in the presence of Ca^{2+} ions [1,2]. Five mammalian paralogous PAD genes, named *PADI1*, *PADI2*, *PADI3*, *PADI4* and *PADI6*, have been described. They are clustered on human chromosome locus 1p35-36 [3]. The encoded PAD isoforms display largely related amino acid sequences, but different tissue-specific expression patterns have been revealed [3–5]. In human epidermal cells, keratins, filaggrin and trichohyalin have been shown to be deiminated during the terminal stages of epidermal differentiation [6–8]. Although the exact physiological functions of protein deimination have not been clarified, the modification dramatically alters the charge of the targeted residues from positive to neutral, probably resulting in the unfolding of the deiminated proteins. Recently, abnormal deimination of keratin K1 has been observed in cutaneous diseases, i.e. psoriasis and bullous congenital ichthyosiform erythroderma [9,10], suggesting an important role for PADs in homoeostasis of the epidermis.

Three PAD isoforms (PAD1, PAD2 and PAD3) are reported been expressed in human epidermis [4,11,12], two of them (PAD1 and PAD3) being also detected in hair follicles [13,14]. Recently, we reported the precise cellular localization of the three isoforms in the human and murine epidermis, and in human anagen hair follicles by confocal and immunoelectron microscopy analyses with antipeptide antibodies highly specific for each of them [5,14,15]. PAD1 was detected in the entire epidermis, including the SC (stratum corneum), PAD2 in all the living layers, whereas PAD3 expression was shown to be restricted to the granular layer and lower SC. Moreover, PAD1 and PAD3 were observed to be co-located with filaggrin in the lower SC, whereas PAD2 accumulates at the keratinocyte periphery in the stratum granulosum. PAD1 and PAD3 were also detected in the inner root sheath, whereas PAD3 was the only isoform expressed in the medulla of the hair follicles. Moreover, in the inner root sheath, PAD3 is co-located with trichohyalin [12,14,16].

These different patterns of location strongly suggest that the expression of each *PADI* gene is tightly controlled during keratinocyte differentiation, as it is in other cell types [17]. In order to understand the regulatory mechanisms involved, we are currently focusing on the characterization of the proximal promoters of the *PADI* genes and on transcription factors bound to these control elements in human keratinocytes. Our previous studies revealed that the human *PADI2* gene lacks canonical TATA and CAAT boxes, and that Sp1/Sp3 (specificity protein 1/3) transcription factors co-operate to control its transcription in keratinocytes [18]. In the present study, we cloned the 5′-flanking region and identified a minimal promoter sequence of the human

Abbreviations used: ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-Y, nuclear factor Y; NF-YA, NF-Y subunit; NHEK, normal human epidermal keratinocyte; PAD, peptidylarginine deiminase; RT, reverse transcription; SC, stratum corneum; siRNA, small interfering RNA; Sp, specificity protein; TBP, TATA-box-binding protein; TE, Tris/EDTA; TFIID, transcription factor IID.

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PADI3 gene. We also report that NF-Y (nuclear factor Y) and Sp1/Sp3 transcription factors bind to the proximal promoter to regulate the expression of *PADI3*.

MATERIALS AND METHODS

Cell cultures

NHEKs (normal human epidermal keratinocytes) were obtained from Clonetics and were cultured in a serum-free keratinocyte growth medium (KGM2; Clonetics) with either 0.15 mM (proliferating conditions) or 1.2 mM Ca^{2+} (differentiating conditions), as described previously [19]. The immortalized keratinocyte line HaCaT, a gift from Professor N. Fusenig (German Cancer Research Center, Heidelberg, Germany), was grown in DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL) supplemented with 5% (v/v) FBS (foetal bovine serum; HyClone Laboratories). HeLa cells were obtained from the Health Sciences Research Resources Bank (Osaka, Japan) and maintained in DMEM with 10% (v/v) FBS. All of the cells were incubated in humidified incubators at 37 °C and 5% CO_2. After reaching 70–80% confluence, the cells were collected for subculture or used for transient transfection.

Analysis of the expression of *PADI3* in cultured cells

The relative expression values of *PADI3* were analysed by real-time RT (reverse transcription)–PCR, as described previously [18]. Primer sequences for real-time RT–PCR studies were as follows: *PADI3* forward primer 5′-AATGTTTGAGGTCTATGGGA-3′ [corresponding to nucleotides 155–174 of the human PAD3 cDNA (complementary DNA), GenBank® accession number AB026831] and reverse primer 5′-CCAAAGTCGCGTCAAAGC-3′ (nucleotides 247–264); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward primer 5′-CATGTTCCAATATGATTCCAC-3′ (nucleotides 187–207 of human GAPDH cDNA, GenBank® accession number M33197) and reverse primer 5′-CCTGGAAGATGGTGATG-3′ (nucleotides 271–287). The amplification programme consisted of denaturation at 95 °C for 3 min, followed by 50 cycles at 95 °C for 30 s and at 59 °C for 30 s using a two-step protocol.

Cloning of the 5′-flanking regions of *PADI3*

Since no human genomic contig covered the nucleotide sequence of the 5′-flanking region of *PADI3* at the beginning of the present study, we screened a genomic library with a 261-bp probe corresponding to the 5′-flanking region (nucleotides 24–284) of the human *PAD3* cDNA. A positive clone (λhPAD3) was isolated, and the corresponding insert was subcloned into pBluescript II SK(+) (Stratagene). The insert, approx. 17 kb, covered exons 1 and 2, and part of the second intron of *PADI3*, but only a short part (725 bp) of the 5′-flanking region of the gene. Therefore we carried out inverse PCR for the amplification of the additional 5′-flanking region. First, we digested human genomic DNA (BD Biosciences) with HindIII and then submitted the DNA fragments to self-ligation with the TaKaRa DNA ligation kit version 1. The resulting circular DNAs were used as templates for PCR amplification. PCR was performed with a pair of specific primers: 5′-GGGCTCGAGTGGGCTTTCATGACTGGAGC-3′ (forward; nucleotides −95 to −75; position number +1 corresponding to the transcription initiation site; underlining shows the recognition sequence for the restriction enzyme XhoI) and 5′-TTGCTTCCTCACCTCCATTTTCTGC-3′ (reverse; nucleotides −323 to −299), and using the Expand™ Long Template PCR system (Roche). After denaturation at 92 °C for 2 min, the PCR was run for 30 cycles with the following programme: 10 cycles of 92 °C for 10 s, 65 °C for 30 s and 68 °C for 15 min, and 20 cycles of 92 °C for 10 s, 65 °C for 30 s and 68 °C for 15 min supplemented with an extension period of 20 s for each cycle; this was followed by a final extension step at 68 °C for 7 min. The amplified PCR product (2044 bp) was cloned into dT-tailed vectors prepared from pBluescript II SK(+) [20]. The plasmid obtained was designated pInvhPAD3. The λhPAD3 and pInvhPAD3 inserts overlapped and covered 2377 bp of the 5′-flanking region of *PADI3*. Their sequences were consistent with those of the *PADI3* gene reported by Chavanas et al. [3] (GenBank® accession number AJ549502).

Sequencing of each clone was carried out using a Big Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) on automated DNA sequencing (373 DNA Sequencer, Applied Biosystems). All sequences thus obtained were analysed with the GENETYX-MAC software (Software Development Co., Tokyo, Japan).

RNase protection analysis

To determine the 5′-flanking end of *PADI3* mRNA, RNase protection assays were performed as described by Zhang et al. [21]. To construct the template plasmid for synthesis of the riboprobe, a *PADI3* fragment containing the transcription initiation site (−129 to +130) was subcloned into the pT7Blue vector (EMD Biosciences). This DNA fragment was prepared by PCR, using clone λhPAD3 as a template with forward primer 5′-CAGCCAATCCTGAGCTC-3′ and reverse primer 5′-ATAAATGTCCACGAGGGTCTCCAC-3′. The resulting construct (pT7-PAD3) was linearized with BamHI, and antisense RNA was synthesized and labelled with biotin from the T7 promoter of the plasmid using the *In Vitro* Transcription kit (BD Biosciences). Total RNA (30 μg) was obtained from NHEKs cultured in the high-calcium medium (1.2 mM) or HaCaT cells with a RNeasy Protect Kit (Qiagen) and was hybridized to the antisense RNA for 16 h at 42 °C using a RNase protection assay kit (BD Biosciences) according to the manufacturer's instructions. Salmon tRNA was used as a negative control. After RNA samples had been digested with an RNase mixture, the sizes of protected RNAs were determined using denaturing 5% (w/v) PAGE using a RiboQuant Non-Rad Detection kit (BD Biosciences). The reverse primer 5′-labelled with FITC was used to perform the cDNA extension of the protected RNA segments using PowerScript reverse transcriptase (BD Biosciences). Using pT7-PAD3 as the template DNA, a sequence reaction was carried out using the Thermo Sequenase Cycle Sequencing kit (USB) and a 7 M urea/10% PAGE run for 2 h at 1500 V with 1 × TBE (Tris/borate/EDTA). The flush signal was detected through the Fluor Imager 595 system (Amersham Biosciences).

Construction of promoter reporter plasmids

To construct the reporter plasmid pG3 − 2317/+41 which covers −2317 to +41 bp from the transcription start site of *PADI3*, we first amplified two DNA fragments of the 5′-upsteam (1838 bp) and 3′-downstream regions (550 bp); the fragments were digested, ligated to each other, and then cloned into the pGBasic vector 2 (Nippon Gene) containing the firefly luciferase gene (*LucF*) but no regulatory elements. In brief, the 5′-upstream region was amplified by PCR, using pInvhPAD3 as a template with forward primer G3−2317/−2288 (Table 1) and reverse primer 5′-CTGGAATTCAATGGTGCGATCTCGG-3′ (nucleotides −515 to −494; underlining shows an EcoRI site) using the following programme: initial denaturation at 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 4 min, and a final extension at 72 °C for 8 min. The 3′-downstream region was

Table 1 Primers used for preparing the reporter plasmids and oligonucleotides for the EMSA

Underlined regions are the recognition sequences for the restriction enzyme XhoI (CTCGAG), BamHI (GGATCC), HindIII (AAGCTT) or NspV (TTCGAA).

(a) Primers for preparing the deletion constructs and site-directed mutagenesis

Name	Sequence	Position
G3 − 2317/− 2288 (forward)	5′-GGGCTCGAGCTAAGTTACCTGGCTTCAGT-3′	− 2317/− 2288
G3 − 1881/− 1862 (forward)	5′-GGGCTCGAGGAAAAAAGTGAAGCTGTCCC-3′	− 1881/− 1862
G3 − 1482/− 1463 (forward)	5′-GGGCTCGAGCCCCAGAGCCCTTGCTCCTG-3′	− 1482/− 1463
G3 − 1057/− 1035 (forward)	5′-GGGCTCGAGTAGCCGGGAGCCGTGGCAGG-3′	− 1057/− 1035
G3 − 657/− 638 (forward)	5′-GGGCTCGAGAGCCTGACCAACATGGAAAA-3′	− 657/− 638
G3 − 276/− 257 (forward)	5′-GGGCTCGAGTATATCTCTGCACCCCCTCA-3′	− 276/− 257
G3 − 129/− 113 (forward)	5′-TAACTCGAGCAGCCAATCCTGAGCTC-3′	− 129/− 113
G3 − 118/− 99 (forward)	5′-TAACTCGAGCTCTCAGCTCTGGGAAG-3′	− 118/− 99
G3 − 100/− 84 (forward)	5′-TAACTCGAGAGGGGCTGGGCTTTCTC-3′	− 100/− 84
G3 − 94/− 75 (forward)	5′-GGGCTCGAGTGGGCTTTCATGACTGGAGC-3′	− 94/− 75
G3 − 65/− 49 (forward)	5′-TAACTCGAGGCACCCTGGGCTGGAG-3′	− 65/− 49
G3 + 21/+ 41 (reverse)	5′-GGGAAGCTTGCTGGTGTTGGACTTAGCTG-3′	+ 21/+ 41
G3 − 48/− 37 mutant (forward)	5′-TAAGGATCCGGCCAGGGGCTTTTAA-3′	− 48/− 37
G3 − 62/− 43 mutant (reverse)	5′-TAAGGATCCCTCCAGCCCAGGGT-3′	− 62/− 43
G3 − 31/− 13 mutant (forward)	5′-TACTTCGAACCTCATCCTAAGG-3′	− 31/− 13
G3 − 43/− 26 mutant (reverse)	5′-TACTTCGAAAGCCCCTGGCCC-3′	− 43/− 26

(b) Oligonucleotides for the EMSA

Name	Sequence	Position
Probe − 138/− 115S	5′-GGTCCCTTCCAGCCAATCCTGAGC-3′	− 138/− 115
Probe − 134/− 111AS	5′-GAGAGCTCAGGATTGGCTGGAAGG-3′	− 134/− 111
Probe − 109/− 85S	5′-GCTCTGGGAAGGGGCTGGGCTTTCA-3′	− 109/− 85
Probe − 104/− 80AS	5′-GGTCATGAAAGCCCAGCCCTTCCC-3′	− 104/− 80
Probe − 81/− 55S	5′-CTGGAGCCAATGAATTGGCACCCTGGG-3′	− 81/− 55
Probe − 77/− 53AS	5′-AGCCCAGGGTGCCAATTCATTGGCT-3′	− 77/− 53

amplified using λhPAD3 as a template with forward primer 5′-ATTGAATTCCAGCCTGGGCAACAAG-3′ (nucleotides − 499 to − 478; underlining shows an EcoRI site) and reverse primer G3 + 21/+ 41 (Table 1) in the same conditions. These amplified PCR products were digested with XhoI/EcoRI for the 5′-upstream region or with EcoRI/HindIII for the 3′-downstream region, ligated to each other at the EcoRI site, and cloned into the XhoI and HindIII sites of pGBasic vector 2. Sequential 5′-deletion constructs of the 5′-flanking region of *PADI3* were generated, as described previously [18], by PCR using pG3− 2317/+ 41 as a template and the primers listed in Table 1. The resulting amplification products were cloned into the XhoI and HindIII sites of pGBasic vector 2. We also constructed two reporter plasmids with nucleotide substitutions altering the consensus sequence of Sp-1 (nucleotides − 226 to − 238) or TFIID (transcription factor IID)-binding sites (nucleotides − 238 to − 252). For substitutions at nucleotides − 226 to − 238, two separate PCR fragments were generated with either a pair of forward G3 − 129/− 113 and reverse G3 − 62/− 43 mutant primers or a pair of forward G3 − 31/− 13 mutant and reverse G3 + 21/+ 41 primers using pG3 − 2317/+ 41 as a template. The products of PCR were digested with XhoI/BamHI for the 5′-upstream region or BamHI/HindIII for the 3′-downstream region, ligated to each other at the BamHI site, and then cloned into the XhoI and HindIII sites of pGBasic vector 2. For the substitutions at − 238 to − 252, we used a pair of forward G3 − 129/− 113 and reverse G3 − 43/− 26 mutant primers or forward G3 − 31/− 13 mutant and reverse G3 + 21/+ 41 primers. The PCR products were digested with XhoI/NspV for the 5′-upstream region or with NspV/HindIII for the 3′-downstream region, ligated to each other at the NspV site, and then cloned into the XhoI and HindIII sites of pGBasic vector 2. All of the plasmids were prepared using the QIAfilter Plasmid Midi kit (Qiagen), and their nucleotide sequences were confirmed by double-stranded DNA sequencing.

Transfection and measurement of promoter activity

Transfection and measurement of promoter activities were carried out following procedures described previously [18]. Four transfections were carried out independently for each construct, and the results were expressed as means ± S.D.

EMSAs (electrophoretic mobility-shift assays)

Three double-stranded oligonucleotide probes were prepared by annealing the following pairs of single-strand oligonucleotides (Table 1): Probe − 138/− 115S and Probe − 134/− 111AS, Probe − 109/− 85S and Probe − 104/− 80AS, and Probe − 81/− 55S and Probe − 77/− 53AS. A fourth double-stranded probe (nucleotides − 65 to + 41) corresponded to the XhoI/HindIII fragment of the pG3− 65/+ 41 plasmid. These probes were labelled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (Nippon Gene). Nuclear extracts were prepared from cultured cells as described by Andrews and Faller [22]. Each reaction mixture (12.5 μl) containing 4 μg of nuclear proteins, 10 mM Hepes/KOH (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10 % (v/v) glycerol, 5 mM PMSF, 25 ng of aprotinin, 25 ng of pepstatin, 25 ng of leupeptin, 1 mM sodium orthovanadate, 2 μg of poly(dI-dC) · (dI-dC) and 5 ng of ^{32}P-labelled probe (1×10^5 c.p.m.) was incubated at 27 °C for 30 min. For supershift experiments, 2 μg of anti-NF-YA (NF-Y subunit), anti-Sp1 or anti-Sp3 antibodies (Santa Cruz Biotechnology) were added to the binding reaction mixture and were incubated on ice for 20 min before addition of the labelled probe. We also carried out a competitive experiment with a synthesized

double-stranded oligonucleotide corresponding to the consensus sequence for TFIID binding (Santa Cruz Biotechnology). DNA–protein complexes were resolved by 4.5 % PAGE in 0.25 × TAE (Tris/acetate/EDTA) and then autoradiographed.

ChIP (chromatin immunoprecipitation) assays

ChIP assays were performed as described by Hung et al. [23] with some minor modifications. Briefly, cultured cells grown on 10-cm-diameter dishes were cross-linked by adding 0.5 % (w/v) formaldehyde in PBS for 15 min at room temperature (25 °C). Cells then were washed three times with ice-cold PBS and were collected by scraping with lysis buffer containing 10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5 % (v/v) Nonidet P40 and Complete™ protease inhibitor cocktail (Roche). Nuclei were collected by centrifugation at 1500 ***g*** for 5 min, resuspended in 50 mM Hepes buffer, pH 7.5, containing 140 mM NaCl, 1 % (v/v) Triton X-100 and Complete™ protease inhibitor cocktail, and then incubated at 4 °C for 30 min. The lysates were sonicated on ice to an average length of 500–1000 bp of chromosomal DNA and subjected to centrifugation at 17 800 ***g*** for 10 min to remove debris. Supernatants were collected and approx. 1 % of this chromatin solution was saved to quantify the amount of input DNA present in different samples before immunoprecipitation. The rest of the chromatin solutions were immunocleared with 10 μl of Protein A/G–agarose slurry (Santa Cruz Biotechnology) with 15 μg of salmon sperm DNA. Immunoprecipitation was performed overnight with agitation at 4 °C with 5 μg of normal IgG, anti-Sp1, anti-Sp3 or anti-NF-YA antibodies (Santa Cruz Biotechnology). Immunoprecipitated chromatin complexes were collected with 20 μl of Protein A/G–agarose slurry for 1 h at 4 °C with agitation. Beads were then washed six times in TE (Tris/EDTA) buffer and extracted twice with TE buffer containing 1 % SDS and 0.1 M $NaHCO_3$. Cross-links were reversed at 65 °C for 12 h, and proteins were digested with proteinase K (0.5 mg/ml) for 2 h at 50 °C. DNA fragments were purified by phenol/chloroform/3-methylbutan-1-ol (25:24:1) and ethanol precipitation, and reconstituted in 50 μl of TE buffer. The DNA solution was analysed by conventional PCR and quantitative PCR. The following primers were used for ChIP PCR analysis: 5′-TCCAGCCAATCCTGAGCT-3′ (forward), and 5′-ATGAGGTTTAAAAGCCCC-3′ (reverse) to amplify promoter region from nucleotides −131 to −2; 5′-AATGTTTGAGGTCTATGGGA-3′ (forward) and 5′-CCAAAGTCGCGTCAAAGC-3′ (reverse) to amplify exon 2 region corresponding to nucleotides 155–264 of the human PAD3 cDNA. The amplification programme consisted of denaturation at 95 °C for 3 min, followed by 50 cycles of 95 °C for 15 s, 56 °C for 10 s and 72 °C for 10 s.

siRNA (small interfering RNA)-based inhibition

NHEKs were transfected using the siRNA transfection reagent (Santa Cruz Biotechnology) with 100 nM siSp1, siSp3, siNF-YA, or siControl (Santa Cruz Biotechnology) according to the manufacturer's instructions. After culturing in antibiotic-free medium for 36 h, total RNA was extracted and analysed by quantitative real-time RT–PCR as described previously [18]. To confirm the specific inhibitory activity of each siRNA, we carried out Western blotting analyses with the antibodies against each transcription factor. Nuclear extracts were prepared from untreated or siRNA-transfected cells as described previously [22]. After incubation at 95 °C for 5 min with loading buffer, nuclear extracts (15 μg) were separated by SDS/PAGE (12.5 % gel), and the level of Sp1, Sp3 or NF-YA was examined by Western blot analysis. Primary anti-Sp1, anti-Sp3, anti-NF-YA or anti-actin antibody (Santa Cruz Biotechnology) was added in skimmed milk solution at a 1:1000 dilution. The secondary anti-goat and anti-rabbit (Santa Cruz Biotechnology) antibodies were used at a 1:5000 dilution. The proteins were detected using ECL® (enhanced chemiluminescence) plus Western blotting detection system (Amersham Biosciences).

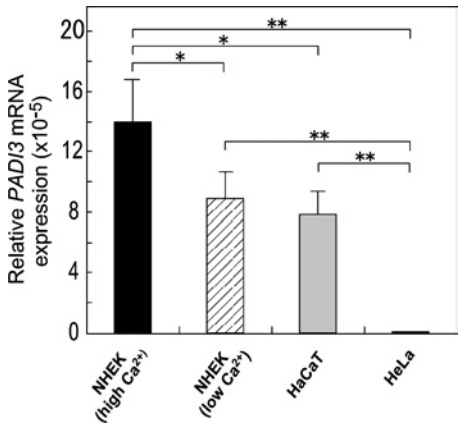

Figure 1 Expression of *PADI3* gene transcripts in NHEKs cultured in high (1.2 mM) or low (0.15 mM) Ca^{2+} ion-containing medium, and in human keratinocyte HaCaT and HeLa cell lines

Total RNAs were obtained from semi-confluent cell cultures. The relative *PADI3* mRNA levels were determined in cDNA samples by quantitative real-time PCR as described in the Materials and methods section. The relative expression values (x) of *PADI3* in cultured cells were calculated using the following equation: $x = 2^{-\Delta Ct}$, where ΔCt is the difference between the Ct values (mean threshold cycle) of *PADI3* and the reference gene (GAPDH). Results are means ± S.D for four separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ (paired Student's t test).

RESULTS

PADI3 expression in cultured human cells

The expression of *PADI3* mRNA was analysed in NHEKs cultured under high (1.2 mM) or low (0.15 mM) Ca^{2+} concentration, and with HaCaT and HeLa cell lines to evaluate their suitability for the analysis of the proximal promoter of the *PADI3* gene and its transcriptional regulation. As shown in Figure 1, quantitative real-time RT-PCR analyses performed on total RNAs isolated from the human cells showed that *PADI3* is expressed in NHEKs and HaCaT cells, but not in HeLa cells: the observed levels were highest in NHEKs cultured under high Ca^{2+} concentration, and a little lower in NHEKs cultured under low Ca^{2+} concentration and in HaCaT cells. These cultured cells were then used for the study of *PADI3* promoter activity.

Identification of the transcription initiation site

In order to determine the transcription initiation site for *PADI3* mRNA, RNase protection analysis was carried out. As shown in Figure 2(A), a single protected RNA segment of 129 nt was produced in HaCaT cells and NHEKs cultured under high-calcium (1.2 mM) medium. Primer extension products from the protected RNA segments indicated that the transcription initiation site was 41 bp upstream of the translation initiation codon (Figure 2B). The transcription initiation site is in good agreement with the size of the protected RNA (Figure 2A) and concordant with the result of our 5′-RACE (rapid amplification of cDNA ends) analysis of human PAD3 cDNA [12]. Furthermore, the nucleotide sequence (5′-GCAGTGTT-3′) around the transcription

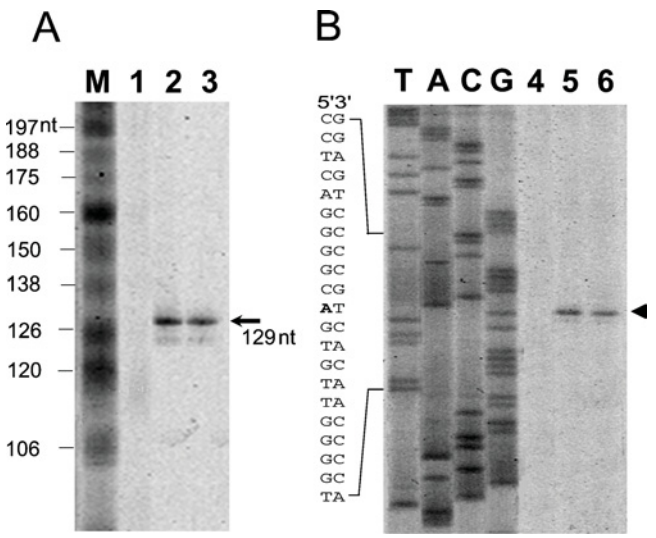

Figure 2 Determination of the transcription start site of the *PADI3* gene

RNase protection analysis was performed on 30 μg of total RNAs from NHEKs (lanes 2 and 5) cultured in high-calcium (1.2 mM) medium and from HaCaT cells (lanes 3 and 6). As a control, 30 μg of tRNA from salmon was used (lanes 1 and 4). (**A**) The protected RNA segments were detected with a 129 nt band (indicated with an arrowhead) using a biotin-labelling method. A sequence reaction of pT7-PAD3 for T with biotin-labelled dUTP was used as molecular-mass marker (lane M). (**B**) Primer extensions were performed using the protected RNA segments as template and FITC-labelled primer as described in the Materials and methods section. The extension products were analysed by electrophoresis in denaturing polyacrylamide gels alongside an adjacent DNA sequencing reaction using the same primer. The primer extension product is indicated with solid arrowhead.

initiation site almost matches the consensus cap sequence 5′-(T/G)C(A/T)(G/T/C)(T/C/A)(C/T)(T/C/G)(T/C)-3′ generally found at nucleotides -2 to $+6$ of eukaryotic promoters [24]. Therefore we determined nucleotide A, 41 bp upstream of the translation initiation codon (ATG), as the transcription initiation site of *PADI3* and referred to it as $+1$.

Identification of the minimal promoter of *PADI3*

To determine which sequences are sufficient to direct transcription of *PADI3*, a 2358 bp fragment (-2317 to $+41$) containing the proximal 5′-flanking region, the transcription start site and a portion of exon 1 was cloned upstream to a promoterless luciferase reporter gene. In addition, progressive 5′ deletions in the putative promoter region were also examined for their effect on the reporter gene activity. The resulting constructs were transiently transfected into NHEKs, HaCaT cells and HeLa cells, and luciferase activities were measured after 48 h. To define putative elements involved in keratinocyte differentiation within the promoter, the constructs were transfected into NHEKs incubated in either low- or high-calcium medium. The results from four independent experiments are shown in Figure 3(A). Significant transcriptional activity was observed with six constructs (pG3 $-$ 2317/$+$41, pG3 $-$ 1881/$+$41, pG3 $-$ 1482/$+$41, pG3 $-$ 1057/$+$41, pG3 $-$ 657/$+$41, pG3 $-$ 276/$+$41). The highest activities were always obtained in NHEKs cultured in high-calcium medium, and moderate activities were observed in NHEKs cultured in low-calcium medium and in HaCaT cells. No significant activities were observed when any of these constructs were transfected into HeLa cells. These results showed that a functional promoter activity was detectable in NHEKs and HaCaT cells, but not in HeLa cells, in agreement with the

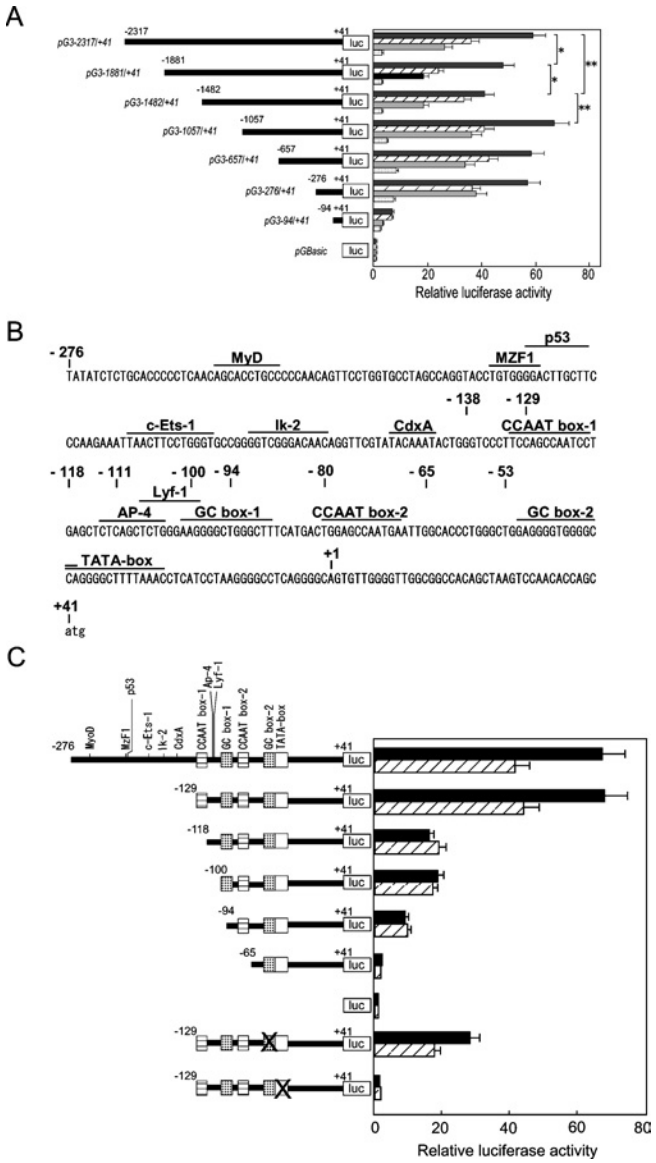

Figure 3 Identification and characterization of the minimal promoter region of the *PADI3* gene

(**A**) Identification of the minimal promoter of *PADI3*. NHEKs cultured in high- (black bar) and low- (hatched bar) calcium medium, and HaCaT (grey bar) and HeLa (dotted bar) cells were transfected with the indicated constructs and assayed for luciferase activity after 48 h. The numbers given to the constructs indicate the 5′- and 3′-ends of the 5′-flanking region of *PADI3*, position numbered $+1$ corresponding to the first base of the transcription initiation nucleotide. Luciferase activity is expressed as fold increase over promoterless vector, pGBasic (set as 1). Values were normalized for transfection efficiency by co-transfection with the *Renilla* expression plasmid, and are expressed as means $\pm$ S.D. for four separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (paired Student's *t* test). (**B**) Sequence and putative transcription factor-binding sites of the minimal promoter of *PADI3*. Numbering of the nucleotides begins with the transcription initiation nucleotide as $+1$. The transcription initiation site was determined by RNase protection assay as shown in Figure 2. Potential transcription factor-binding sites predicted by the GenomeNet MOTIF program (motif library: TRANSFAC; classification: vertebrates; cut-off score: 85, Kyoto University Bioinformatics Center, Kyoto, Japan) are underlined. (**C**) Characterization of the transcription factor-binding sites in the *PADI3* promoter. Shown is the schematic diagram of serial deletion constructs of the *PADI3* promoter and their luciferase activities in NHEKs cultured in high- (black bar) and low- (hatched bar) calcium medium. The numbers given to the constructs indicate the 5′- and 3′-ends of the *PADI3* promoter region. The putative transcription factor-binding sites, which are targeted, are shown on the constructs. Site-directed mutagenesis was carried out with the construct spanning the -129 to $+41$ region. The relative positions of each mutation designated as a cross are shown. See text for additional details. Values were corrected for transfection efficiency by co-transfection with the *Renilla* expression plasmid, and are expressed as means $\pm$ S.D. for four separate experiments.

data on *PADI3* mRNA expression shown in Figure 1. Among the constructs, the plasmid containing fragment −1482/+41 (designated pG3 − 1482/+41) presented a significantly lower activity in NHEKs cultured in high-calcium medium, suggesting the presence of a cell-specific suppressor in the −1881/−1057 region (Figure 3A). Furthermore, truncation of the region between −276 and −94 (to generate pG3 − 94/+41) dramatically decreased the transcriptional activity in any cell type, indicating that this region contained positive regulatory elements that are essential for basal promoter function. Examination of its sequence with the GenomeNet MOTIF program revealed several *cis*-elements for binding of transcription factors, i.e. MyD, MZF1, p53, c-Ets-1, Ik-2, CdxA, AP-4 and Lyf-1 (Figure 3B). In addition, a conventional TATA box at nucleotides −39 to −25, two GC boxes at nucleotides −51 to −38 and −101 to −88, and two CCAAT boxes at nucleotides −80 to −68 and −131 to −120 were identified. The presence of multiple putative transcription factor-binding sites near the transcription initiation site strongly suggests that the region −276/+41 contains the functional minimal promoter of *PADI3*.

Identification of potential *cis*-elements involved in *PADI3* gene regulation

In order to further determine potential *cis*-elements of the minimal promoter involved in the transcriptional control of the expression of *PADI3*, we constructed a new series of deletion mutants. They were transiently transfected into NHEKs cultured in low- or high-calcium medium in addition to the parental construct pG3 − 276/+41 (Figure 3C). Deletion of 147 nucleotides from the 5′-end of the pG3 − 276/+41 construct had no effect on the promoter activity. It is likely that the putative transcription factor-binding sequences identified between nucleotides −276 and −129 are not functional. The luciferase activities observed in NHEKs cultured under in each medium decreased sharply with the deletion of the putative CCAAT box-1 at nucleotides −131 to −120 (pG3 − 118/+41). Moreover, this construction and the further deleted mutants displayed the same relative luciferase activities in keratinocytes cultured in low- and high-calcium medium, suggesting that a differentiation-responsive element might exist within the putative CCAAT box-1. Relative to the activity of the −118/+41 construct, lack of putative Ap-4 and Lyf-1 binding elements showed no effects (pG3 − 100/+41). The luciferase activities observed in NHEKs cultured in each medium gradually decreased with the successive deletions of GC box-1 at nucleotides −101 to −88 and CCAAT box-2 at −80 to −68.

When deletion went further, to construct *pG*3 − 65/+41, the promoter activity was almost completely abolished. As shown in Figure 3(B), the *PADI3* gene contains a second putative GC box-2 and a TATA box close to the transcription initiation start site. Considering the co-operative role of these boxes in gene regulation, the effect of mutations in these putative *cis*-elements on *PADI3* promoter activity was determined using site-directed mutagenesis. Using the construct spanning the region −129 to +41, the core binding site of the GC box-2 was changed from GGGTGG (at nucleotides −48 to −43) to GGATCC. A mutation in the putative TATA box also changed the core binding site from TTTAAA (at nucleotides −32 to −27) to TTCGAA. Mutation of GC box-2 eliminated about half of the activity compared with that of the original plasmid, and mutation of the TATA box completely abolished *PADI3* promoter activity (Figure 3C). These results strongly suggest that both CCAAT and both GC boxes of the region between −129 to −38 bp are co-operative *cis*-elements critical for *PADI3* promoter activity.

Binding of NF-Y, Sp1 and Sp3 to the *cis*-acting elements

Having shown that the CCAAT and GC boxes upstream of the *PADI3* gene are necessary for its transcription, we next performed an EMSA to identify proteins with the potential to interact with these sites. ^{32}P-labelled double-stranded oligonucleotide probes containing either of the boxes were incubated with nuclear extracts from NHEKs, and HaCaT and HeLa cells. Figures 4(A) and 4(C) show that both elements containing a CCAAT box (−138/−111 and −81/−53) apparently formed the same two protein–DNA complexes with each nuclear extract used. The intensities of the complexes are very similar when comparing the extracts of HeLa and HaCaT cells and NHEKs. Since NF-Y is the major protein that recognizes the CCAAT box [25], we used antibody against NF-YA (one of the three subunits of NF-Y trimer) in an EMSA supershift assay to identify specific proteins involved in the formation of the complexes. The addition of anti-NF-YA antibody resulted in the disappearance of one of the two complexes and in the appearance of a new supershifted band, providing evidence for the involvement of NF-Y. However, one of the retarded bands (indicated as unknown in Figures 4A and 4C) was not supershifted even in the presence of sufficient antibody, suggesting that other transcription factor(s) with a binding specificity similar to that of NF-Y was present in the nuclear extracts. Specific gel shifts were also obtained when nuclear extracts from the cultured cells were assayed with radiolabelled oligonucleotides corresponding to the GC box-1 (−109/−80). As shown in Figure 4(B), three major shifted complexes were observed that could be supershifted with antibodies against Sp1 and Sp3. Sp3 is usually expressed either as a full-size or as an N-terminally truncated form without a complete DNA-binding activity [26]. This probably explains why two bands were supershifted by the anti-Sp3 antibody: the upper band corresponding to the entire transcription factor and the lower band corresponding to the truncated form (indicated by Sp3′ in Figure 4B). The relative ratios of the three bands were very similar when comparing the extracts of HeLa, HaCaT and NHEKs, but their intensity seemed to be greater when using extracts of NHEKs. We also observed a fourth faint band with high mobility (indicated by unknown in Figure 4B). Since its intensity was almost the same with each nuclear extract, it therefore seems to be due to non-specific binding. To determine whether the second GC box (GC box-2) and the TATA box (−51 to −25) critical for *PADI3* promoter activity were actually occupied by DNA-binding proteins in nuclei of the cultured human cells, we prepared radiolabelled double-stranded oligonucleotides containing both elements and performed EMSAs (Figure 4D). The profiles of retarded bands resembled those found using the region −109/−80 as a probe (see Figure 4B). The protein–DNA complexes were clearly more abundant when using extracts of NHEKs and HaCaT cells than the extract of HeLa cells (Figure 4D, panel 1). Similar experiments performed with the specific antibodies against Sp1 or Sp3 revealed that three retarded bands contained Sp1, Sp3 and the truncated Sp3 (Sp3′) (Figure 4D, panel 2). As shown in this Figure, a retarded band was not supershifted by the addition of anti-Sp1 or anti-Sp3 antibodies (indicated by an open arrow). Competition experiments with an unlabelled probe corresponding to the consensus TFIID-binding site (TATA box) confirmed that TFIID is involved in the formation of this complex (Figure 4D, panel 2, compare lanes 5 and 6 with lane 4). Taken together, these results suggest that the binding of NF-Y to the two CCAAT boxes and the binding of Sp1 and Sp3 to the two GC-boxes in the minimal promoter region of the *PADI3* gene are critical events to control its transcription in human keratinocytes.

To test whether Sp1/Sp3 and NF-Y proteins actually bind to the *PADI3* promoter *in vivo*, we performed a ChIP assay using

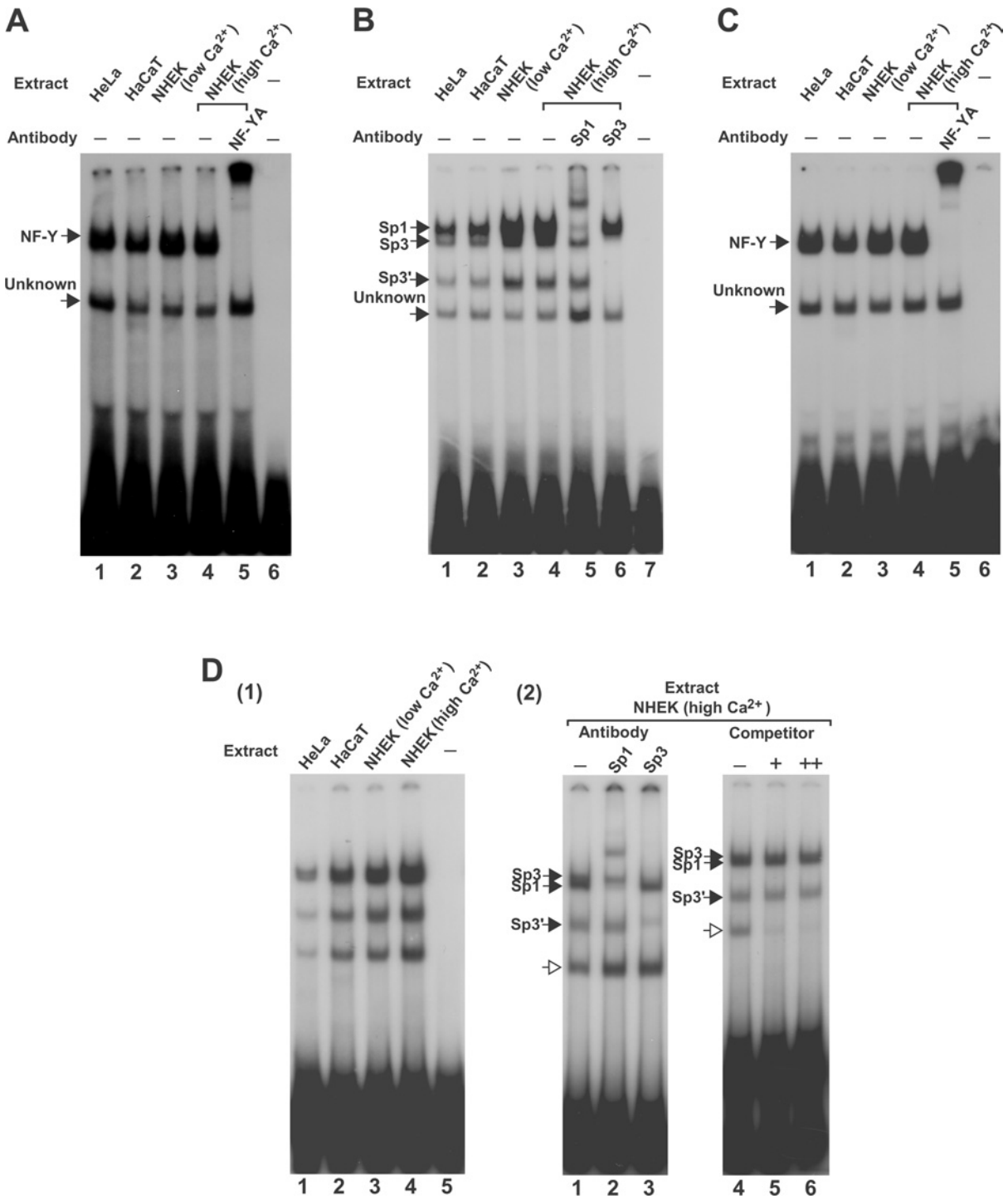

Figure 4 Binding of NF-Y, Sp1 and Sp3 to the *cis*-acting elements of the *PADI3* promoter

EMSAs were performed using synthetic oligonucleotides or a plasmid fragment corresponding to regions containing putative transcription factor-binding elements, and nuclear extracts (4 μg of protein) of HeLa and HaCaT cells, and NHEKs cultured in low- and high-calcium medium. For supershift assays, EMSA was performed using specific antibodies against transcription factors and nuclear extracts of NHEKs cultured in high-calcium medium. For controls, each EMSA with only the labelled probe was carried out. Sequences of the probes used are given in Table 1 or in the Materials and methods section. (**A**) EMSA using the labelled probes −138/−111 and supershift assays with the probes and antibodies against NF-YA. (**B**) EMSA using the labelled probes −109/−80 and supershift assays with the probes and anti-Sp1 or anti-Sp3 antibodies. (**C**) EMSA using the labelled probes −81/−53 and antibodies against NF-YA. (**D**) Panel 1: EMSA using the labelled probes −65/+41. Panel 2: supershift assays with the labelled probes −65/+41 and anti-Sp1 or anti-Sp3 antibodies and competitive experiments with a synthesized double-stranded oligonucleotide having a consensus sequence for TFIID binding. The molar ratios of the competitive DNA to the labelled probes were 0 (lane 4), 10-fold (lane 5) and 50-fold (lane 6) respectively.

specific antibodies directed against Sp1, Sp3 or NF-YA, primers specific for the *PADI3* promoter region and formaldehyde-fixed chromatin isolated from cultured cells. The results shown in panel 1 of Figure 5(A) indicate that Sp1, Sp3 and NF-YA bind to the 5′-flanking region of *PADI3*. When PCR using the same primers were performed on the chromatin samples

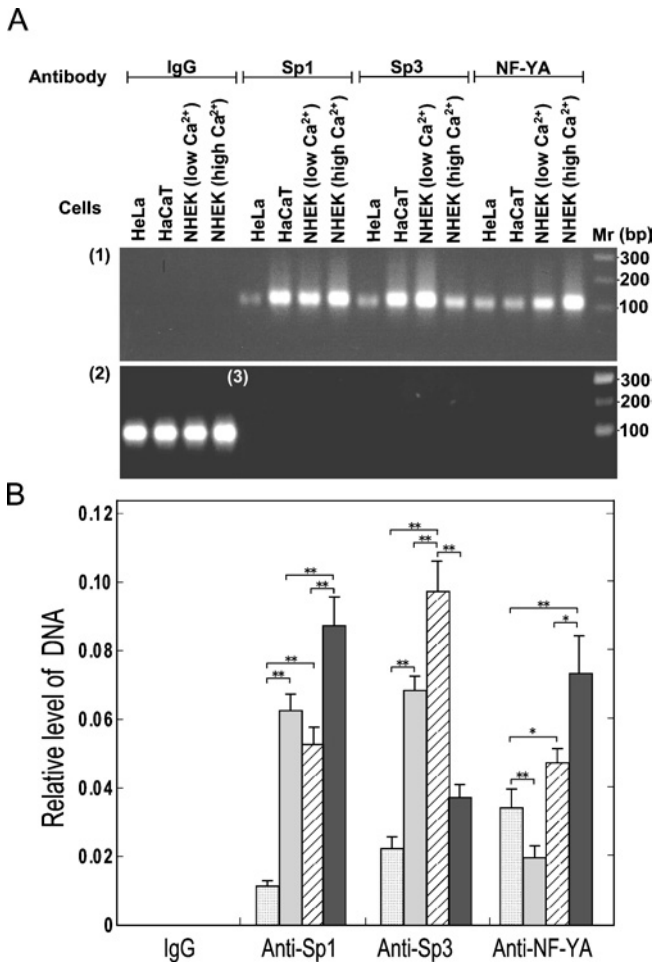

Figure 5 Sp1, Sp3 and NF-Y binding to the *PADI3* promoter *in vivo*

ChIP assays using anti-Sp1, anti-Sp3 or anti-NF-YA antibodies or without any antibodies were performed using chromatin from HeLa and HaCaT cells, and from NHEKs cultured in low- and high-calcium medium as described in the Materials and methods section. (**A**) Sp1, Sp3 or NF-Y binding to the *PADI3* promoter was detected by gel staining after PCR amplification using primers corresponding to the promoter region (panel 1) and exon 2 region of *PADI3* gene (panel 3). Results of control PCR, using the primers on the promoter region and input DNA as template, are shown in panel 2. Sizes are given in bp. (**B**) For quantitative analyses of Sp1, Sp3 or NF-Y binding to the *PADI3* promoter, the samples from HeLa (dotted bar) and HaCaT (grey bar) cells and from NHEKs cultured in low- (hatched bar) and high- (black bar) calcium medium were analysed using real-time PCR. The relative DNA levels were calculated as described in the Materials and methods section. Results are means ± S.D. for four separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (paired Student's *t* test).

immunoprecipitated with non-immune IgG, no PCR signal was observed, showing the specificity of binding. The input levels of chromatin for each cell line were shown by the PCR using input DNA as templates (Figure 5A, panel 2). Further specificity of the ChIP analysis was demonstrated by the inability to detect binding of Sp1/Sp3 or NF-Y to the exon 2 region of *PADI3* gene using the primers used in RT–PCR (Figure 5A, panel 3). To quantify apparent cell-specific differences in transcription factor binding, we carried out quantitative PCR with the immunoprecipitated chromatin. As shown in Figure 5(B), the highest levels of Sp1 or/and Sp3 recruitment were observed in NHEKs and HaCaT cells. Interestingly, the ratio of Sp1 to Sp3 binding was reversed when NHEKs were cultured under high- (differentiation condition) compared with low- (proliferation condition) calcium medium, from 2.3:1 to 1:1.8 respectively. The magnitude of NF-YA recruitment also varied among the different cells and culture conditions: the level of binding being highest in NHEKs under high Ca^{2+} ion concentration (Figure 5B). These findings suggest that the association of NF-Y and Sp1 on the promoter region of *PADI3* may play a prominent role in the transcription of the gene.

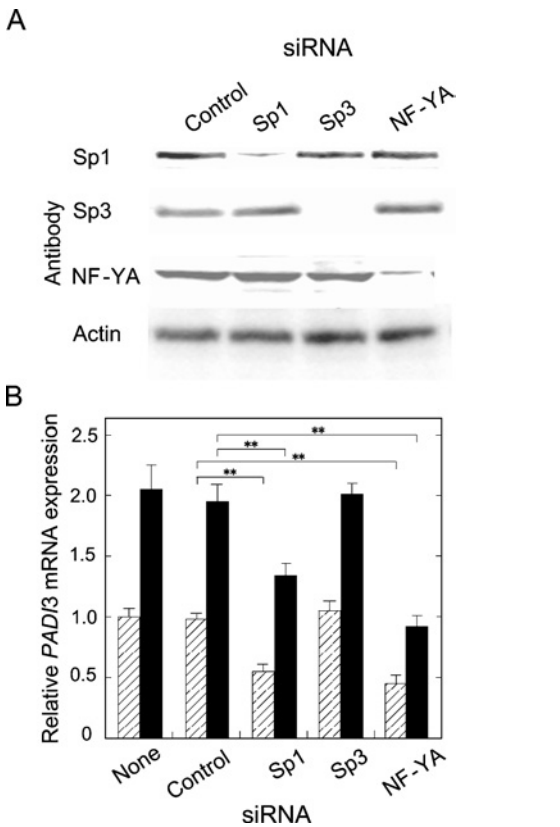

Figure 6 Binding of Sp1 and NF-Y to the *PADI3* promoter is essential for *PADI3* expression in NHEKs

NHEKs were transfected with 100 nM of the indicated siRNA and cultured for 36 h. Nuclear proteins and total RNA were extracted for Western blotting with the indicated antibodies, and quantitative real-time PCR analysis respectively. (**A**) Inhibition of expression of Sp1, Sp3 and NF-YA with their respective specific siRNA was confirmed by Western blotting, compared with control siRNA treatment. The protein levels of the nuclear isoform of actin were used as loading control. (**B**) Effects of partial inhibition of Sp1, Sp3 and NF-YA on *PADI3* expression. *PADI3* expression level in NHEKs cultured in low- (hatched bar) and high- (black bar) calcium medium was normalized to the level of expression of GAPDH, and expressed relative to the non-treated NHEKs cultured in low-calcium medium. Results are means ± S.D. for three experiments. $^{**}P < 0.01$ (Student's *t* test).

Furthermore, we carried out siRNA experiments to investigate the role of Sp1, Sp3 and NF-Y *in vivo* for *PADI3* gene regulation. As shown in Figure 6(A), the siRNAs transferred into NHEKs hindered the synthesis of each transcription factor in a specific manner. When Sp1 expression was reduced by specific siRNA transfection, the level of *PADI3* expression in cells cultured in both low- and high-calcium medium was diminished significantly (Figure 6B). When NF-YA expression was reduced by NF-YA-specific siRNA (Figure 6A), the level of *PADI3* expression in NHEKs cultured in low- and high-calcium medium was diminished by over 50 % (Figure 6B). However, when the Sp3 expression level was effectively silenced by Sp3-specific siRNA (Figure 6A), the level of basal and Ca^{2+} ion-induced *PADI3* expression was not significantly affected (Figure 6B). Control siRNA did not significantly affect basal and Ca^{2+} ion-induced *PADI3* expression.

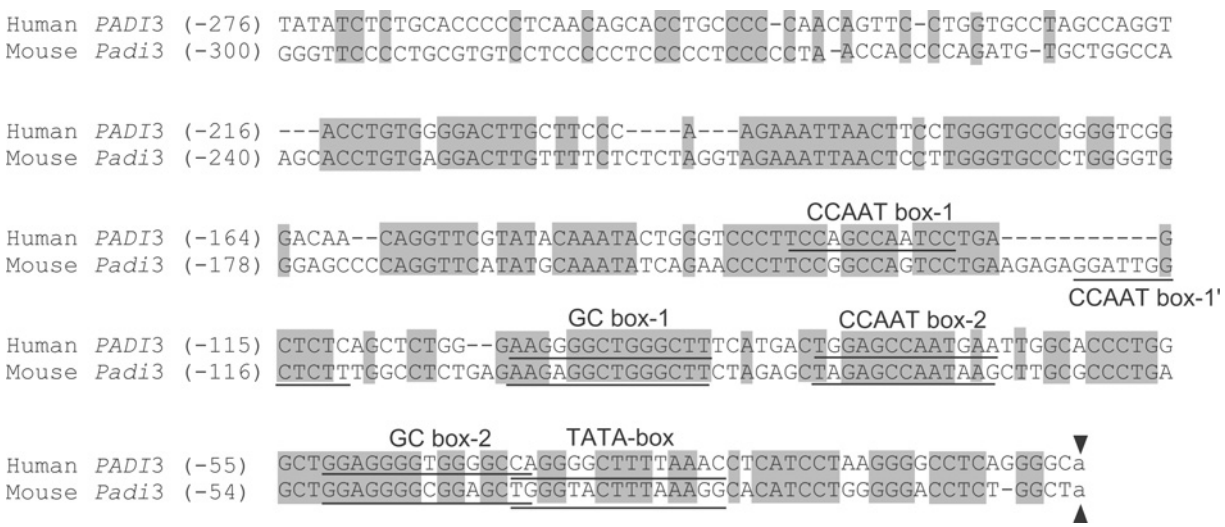

Figure 7 Sequence alignment of the promoter regions of *PADI3* gene and mouse *Padi3* gene

Fragments of 270 nt and 300 nt respectively from 5′ to the transcription start site of *PADI3* sequence (GenBank® accession number AJ549502) and *Padi3* (GenBank® accession number AB121692) were used for alignment, using the ClustalW algorithm from the genetic information processing software (GENETYX-MAC). Grey boxes indicate identity. The lower-case nucleotides with overlying filled arrowheads indicate start sites of transcription for *PADI3* (as described in the text) and *Padi3* (R. Gotoh, T. Kojima, M. Shiraiwa and H. Takahara, unpublished work). The sequences of transcription factor-binding sites are underlined.

DISCUSSION

The transcription initiation site of the *PADI3* gene was mapped 41 bp upstream of the translational start site and was located approx. 20 bp downstream of a putative TATA box. As discussed below, the nucleotide length and sequence from the transcription start site to the TATA box consensus sequence is highly conserved in the murine orthologous gene, *Padi3* (Figure 7). This could indicate the strong evolutionary forces conserving the nucleotide sequences of the promoter region of PAD type III genes. Using the program DNA ClustalW, we carried out comparative analyses to detect conserved nucleotide blocks within the 5′-flanking regions of human and mouse *PAD3* genes. As shown in Figure 7, the minimal promoter region was especially conserved and CCAAT-, GC- and TATA-like boxes were observed. Surprisingly, the core nucleotide sequence of CCAAT box-1 at nucleotides −126 to −122 of human *PADI3* is CCAGT in mouse *Padi3*. As a substitute, a putative CCAAT box-1′ sequence might be inserted into mouse *Padi3* in the opposite direction (−117 to −121). Based on the knowledge of the function of the *cis*-element regardless of the direction of and distance from the promoter [27], we deduced that all of these boxes, highly conserved in human and mouse, are indispensable for *PAD3* gene expression.

Reporter gene assays and EMSAs also revealed that *cis*-elements, important for cell-specific *PADI3* transcription in the minimal promoter region are two GC boxes at nucleotides −101 to −88 and −51 to −48. These boxes showed Sp1- and Sp3-binding activities. Sp1 and Sp3 recognize the same DNA element and are co-expressed in several tissues/cell types [26]. In addition, the Sp1 family has been shown to be involved in inducible gene transcription, such as responses to glucose, serum, epidermal growth factor, platelet-derived growth factor, and transforming growth factor [26]. Furthermore, Sp3 expression can either activate or repress promoter activity, depending on the individual promoter and cell line [28–30]. ChIP assays in the present study indicated that the levels of Sp1 and/ors Sp3 recruitment to the promoter and the Sp1/Sp3 ratio were changed according to cell types, consistent with the mRNA levels of *PADI3* gene in the cells (Figures 1 and 5). When the Sp1 expression level was effectively silenced by siRNA transfection, the level of basal and Ca^{2+} ion-induced *PADI3* expression was significantly diminished (Figure 6). However, silencing of Sp3 expression using specific siRNA did not significantly influence the level of basal and Ca^{2+} ion-induced *PADI3* expression (Figure 6). Thus we believe that Sp1/Sp3 binding provides a mechanism by which transcription of *PADI3* is controlled, and this regulation depends on changes in the Sp1-binding level and Sp1/Sp3 ratio, as we proposed previously for the regulation of *PADI2* gene transcription [18].

The two putative Sp1-binding sites (GC boxes) that we identified in the minimal promoter region of *PADI3* were located between two CCAAT boxes or between a CCAAT box and a TATA box (Figure 3B). Deletion or site-directed mutagenesis of one CCAAT or GC box dramatically decreased the promoter activity, suggesting that a single CCAAT box or GC box by itself enables the transcription of Sp1 and NF-Y [31–36]. The physical interaction between Sp1 and NF-Y has also been demonstrated [37,38]. Furthermore, it was found that NF-Y or Sp1 interacts with TBP (TATA-box-binding protein), which recognizes the TATA box and associates with TFIID for the initiation of gene transcription [39]. The site-directed mutagenesis assays that we have performed also suggest that the putative TATA box at nucleotides −39 to −25 contributes greatly to the promoter activity for *PADI3* expression (Figure 3C). Taken together, these finding strongly support that the association of NF-Y, Sp1/Sp3 and TBP regulates the basal expression of *PADI3*.

We demonstrated that two CCAAT boxes are required for *PADI3* transcription and we showed that the NF-Y transcription factor, present in keratinocyte extracts, actually binds to the corresponding CCAAT box sequences *in vitro* and *in vivo* (Figures 3C, 4A, 4C and 5). Furthermore, when NF-YA expression was specifically reduced by siRNA, the level of basal and Ca^{2+} ion-induced *PADI3* expression was significantly diminished (Figure 6). The levels of NF-Y vary in different cell types or growth conditions [40], and its DNA-binding properties are

influenced by intracellular Ca^{2+} concentrations [41]. Therefore we speculate that the level of intracellular Ca^{2+} in the cultured human cells is sufficient to maintain NF-Y binding to the *PADI3* promoter region. On the basis of these clues, it might be a key issue that the level of Ca^{2+} is changed within the different epidermal layers in human skin to allow an understanding of the differentiation of keratinocytes and the regulation of *PADI3* expression.

In the epidermis, PAD3 is specifically immunodetected from the granular layer [5], whereas, in cultured keratinocytes, its mRNA is significantly detected even in proliferative conditions (similarly to basal keratinocytes). ChIP and siRNA assays suggest that the Sp1/Sp3 ratio and NF-Y-binding levels were involved in the cell-specific expression of *PADI3* gene, but other mechanisms of regulation could be also essential for PAD3 expression *in vivo*, such as control of mRNA translation. The *PADI3* gene might be transcribed from the basal layer but only translated in the granular layer. This apparent discrepancy has also been described for other *PADI* mRNAs, i.e. *PADI2* and *PADI4* in synovial fluid mononuclear cells [17]. It is therefore necessary to investigate further the translational control of *PADI3* to understand the tissue-specific expression of the gene.

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