Regulation of desmocollin gene expression in the epidermis: CCAAT/enhancer-binding proteins modulate early and late events in keratinocyte differentiation

Conrad SMITH*, Kuichun ZHU*, Anita MERRITT†, Rhian PICTON*, Denise YOUNGS*, David GARROD† and Martyn CHIDGEY*[1]

*Division of Medical Sciences, University of Birmingham, Clinical Research Block, Queen Elizabeth Hospital, Birmingham B15 2TH, U.K., and †School of Biological Sciences, University of Manchester, 3.239 Stopford Building, Oxford Road, Manchester M13 9PT, U.K.

Desmocollins (Dscs) are desmosomal cadherins that exhibit differentiation-specific patterns of expression in the epidermis. Dsc3 expression is strongest in basal cell layers, whereas Dsc1 is largely confined to upper, terminally differentiating strata. To understand better the processes by which Dsc expression is regulated in the epidermis, we have isolated *Dsc3* and *Dsc1* 5′-flanking DNAs and analysed their activity in primary keratinocytes. In the present study, we found that transcription factors of the CCAAT/enhancer-binding protein family play a role in the regulation of expression of both Dscs and, in so doing, implicate this class of transcription factors in both early and late events in keratinocyte differentiation. We show that Dscs are differentially regulated by C/EBP (CCAAT/enhancer-binding protein) family members, with Dsc3 expression being activated by C/EBPβ but not C/EBPα, and the reverse being the case for Dsc1. Expression of both Dscs is activated by another family member, C/EBPδ. These results show for the first time how desmosomal cadherin gene expression is regulated and provide a mechanism for the control of other differentiation-specific genes in the epidermis.

Key words: CCAAT/enhancer-binding protein (C/EBP), desmocollin (Dsc), desmosome, epidermis, keratinocyte.

INTRODUCTION

Desmosomes are complex intercellular junctions that play a critical role in the maintenance of normal tissue architecture. Their major constituents belong to three gene families, namely cadherins, armadillo proteins and plakins. There are two desmosomal cadherin subfamilies, namely desmocollins (Dscs) and desmogleins (Dsgs). In humans, three Dscs (Dsc1, Dsc2 and Dsc3) and four Dsgs (Dsg1, Dsg2, Dsg3 and Dsg4), including the recently described Dsg4 [1], have been identified. Two more Dsgs were found to be present in mice [2–4]. Dscs and Dsgs are membrane-spanning glycoproteins that mediate adhesion by interacting with other family members on adjacent cells [5–7]. Within the cell, they associate with armadillo family members, which link the desmosomal cadherins to desmoplakin, a member of the plakin family of cytolinkers. This in turn tethers the entire complex to the intermediate filament cytoskeleton. Linear chains of cadherin-armadillo-plakin molecules probably comprise the backbone of the desmosome, with further complexity provided by lateral protein interactions so that a three-dimensional meshwork is formed [8,9].

Gene targeting of desmosomal cadherins in mice and characterization of some human pathologies have underscored the importance of these molecules. Thus targeted deletion of either *Dsc1* [10] or *Dsg3* [11] causes a loss of keratinocyte cell adhesion in the epidermis, and inactivation of *Dsg2* results in early embryonic lethality [12]. The clinical relevance of the desmosomal cadherins is revealed by the blistering diseases pemphigus foliaceus and pemphigus vulgaris, which are caused by autoantibodies against Dsg1 and Dsg3 respectively, and by the staphylococcal scalded-skin syndrome (Ritter disease), which is caused by the specific cleavage of Dsg1 by exfoliative toxin A [13]. Mutations in human *DSG1* result in the skin disease striate palmoplantar keratoderma [14,15], whereas those in *DSG4* cause hypotrichosis [1].

The desmosomal cadherins show complex patterns of expression. Thus Dsc2 and Dsg2 are ubiquitously expressed in desmosome-producing tissues, whereas Dsc1, Dsc3, Dsg1 and Dsg3 are restricted to stratified epithelia such as the epidermis. In the epidermis, Dscs and Dsgs show characteristic patterns of expression, with the '1' isoforms largely confined to upper, terminally differentiating strata and the '2' and '3' isoforms found in more basally located cell layers [16]. Quantification of Dsc1 and Dsc3 levels in bovine epidermis has shown that the amounts of Dsc1 and Dsc3 are reciprocally graded within the tissue in a manner consistent with linked gene expression [17]. All *Dsc* and *Dsg* genes are clustered on chromosome 18q12.1 [18]; therefore the possibility exists that desmosomal cadherin gene expression is controlled by a common locus control element. The unusual graded distributions of Dscs in the epidermis has led to the suggestion that they may play a role in regulating keratinocyte differentiation [16], and this idea is supported by the results from transgenic mouse experiments in which mis-expression [19,20] or targeted ablation [10] of desmosomal cadherins has resulted in altered patterns of epidermal differentiation. Recent results have also implicated desmosomal cadherins in cell positioning and morphogenesis in the mammary gland [21].

Little is known about how the complex differentiation-specific patterns of desmosomal cadherin expression are achieved in the epidermis. Analysis of numerous epidermal-specific promoters has implicated a common set of transcription factors, including members of the AP-1 (activator protein-1), AP-2 and Sp1 families, in regulating keratinocyte-specific gene expression [22,23]. One possibility is that specialized differentiation-specific factors act in common with common 'generic' factors to regulate gene expression in different cell layers of the skin. Relevant factors, which

Abbreviations used: AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; Dsc, desmocollin; Dsg, desmoglein; EMSA, electrophoretic mobility-shift assay; LIP, liver-enriched transcriptional inhibitory protein; SV40, simian virus 40.

[1] To whom correspondence should be addressed (e-mail M.A.Chidgey@bham.ac.uk).

Table 1 Oligonucleotides used

Number	Sequence	Application
1	GATAAAACTTGACATTTAACTAGGAGGGGCCACCC	Mutation of Dsc3 EBP(− 193)
2	GGCCCCTCCTAGTTAAATGTCAAGTTTTATCACCTCC	Mutation of Dsc3 EBP(− 193)
3	GCAAGCTGCTGCGCATTCCCTCCTGCGTCCAGGC	Mutation of Dsc3 EBP(− 73)
4	GGACGCAGGAGGGAATGCGCAGCAGCTTGCCGAG	Mutation of Dsc3 EBP(− 73)
5	AAGTGGCAGGAGAACGCTGCTTTGAGGAGG	Mutation of Dsc1 EBP
6	AACCCGGGGGTGATAAAACTTGAGAAATAACTAGGAGG	Multimerization of Dsc3
	GGTGATAAAACTTGAGAAATAACTAGGAGGCCCGGGAA	EBP(− 193)
7	ACTGGTACCATGGCGGCCGGTTTCCCG	PCR of C/EBPβ isoform LIP
8	AGTCTCGAGCTAGCAGTGGCCCGCCGAG	PCR of C/EBPβ isoform LIP
9	TTGGTACCAGATTTTCATTTCGCTCCAG	PCR of C/EBPδ
10	AAGAATTCGCAGGTCCCAAAGAAACTAG	PCR of C/EBPδ
11	GTGATAAAACTTGAGAAATAACTAGGAG*	Dsc3 EBP(− 193) EMSA
12	AAGCTGCTGCGGAAACCCTCCTGCGTCC*	Dsc3 EBP(− 73) EMSA
13	GAGAACTTTTGAAAGCTGCTTTGAGGAG*	Dsc1 EBP EMSA

* Oligonucleotides comprise the 5′–3′ strand of double-stranded EMSA binding probes.

result in abnormal differentiation when absent or ectopically expressed in the epidermis, include POU domain proteins [24,25], NF-κB (nuclear factor κB) family members [26] and steroid hormone receptors [27,28]. The C/EBP (CCAAT/enhancer-binding protein) family of transcription factors are known to play a role in the differentiation of a number of tissues, including the epidermis [29], and are good candidates for the differentiation-specific transcriptional regulation of epidermal genes. The C/EBP family is composed of at least six members, namely C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ [30]. Out of these, C/EBPα, C/EPBβ, C/EBPδ and C/EBPζ (CHOP/Gadd153) are expressed in the epidermis and hair follicles [31,32]. C/EBPs are supposed to have a functional role in the regulation of expression of early [29,33] and late [34] differentiation markers. In the present study, we show that C/EBPs play a role in the transcriptional regulation of *Dsc3* and *Dsc1* and thereby confirm the role of this class of transcription factors in both early and late events in keratinocyte differentiation. We present evidence for the differential regulation of these genes by C/EBP proteins, a mechanism that may be of general applicability for the regulation of other genes that are expressed in a differentiation-specific manner in the epidermis.

EXPERIMENTAL

DNA manipulation

All recombinant DNA technology was performed according to standard procedures. Mouse RPCI21 PAC (P1 artificial chromosome; Human Genome Mapping Project Resource Centre, Hinxton Hall, Cambridge, U.K.) and 129SVJ λFixII (Stratagene) genomic libraries were screened with 5′-fragments of the mouse Dsc3 and Dsc1 cDNAs. A *Hin*dIII fragment containing 3.9 kb Dsc3 5′-flanking sequence was isolated from PAC 373l6. Analysis revealed the 3′ *Hin*dIII site to be located 113 bp upstream of the ATG translation-initiation codon. Bases from − 113 to − 1 were amplified by PCR using PAC DNA as the template and a 4 kb fragment of contiguous upstream DNA cloned into plasmid pGL3-Basic (Promega). Overlapping *Pst*I and *Pvu*II fragments containing 5.2 and 2.1 kb respectively of Dsc1 5′-flanking sequence were isolated from λC1 [10]. Analysis revealed the 3′ *Pvu*II site to be located at − 35. Bases − 35 to − 1 were amplified by PCR using λC1 as the template. Contiguous DNA (6.8 kb), consisting of a 6.7 kb *Pst*I–*Pvu*II fragment and bases − 35 to − 1, was cloned into plasmid pGL2-Enhancer (Promega). 5′ Deletions in the Dsc3 and Dsc1 promoters were achieved using either convenient restriction enzyme sites or PCR amplification. Truncated DNA fragments were cloned into pGL3-Basic (Dsc3) or pGL2-Enhancer (Dsc1).

Site-directed mutagenesis was performed using plasmid pDsc3-(293)luc, containing bases − 293 to − 1 of Dsc3 5′-flanking DNA cloned into the *Sma*I and *Xho*I sites of pGL3-Basic, as a template. The Quik Change kit (Stratagene) was used according to the manufacturers' instructions. The core sequence of a putative C/EBP site at base − 193 was mutated (TTGAGAAA to TTGACATT) with primers 1 and 2 (Table 1). Similarly, the core sequence of a possible C/EBP site at − 73 was mutated (TGCGGAAA to TGCGCATT) using primers 3 and 4 (Table 1). After the mutagenesis, the DNA between the S*ma*I and *Xho*I sites was fully sequenced to confirm the presence of the mutation and to discount the possibility of other, unexpected base changes. An *Sma*I–*Xho*I restriction fragment was then used to replace the corresponding restriction fragment in the original template DNA. A similar strategy was used to mutate the putative C/EBP site at − 311 of Dsc1. Plasmid pDsc1(417)luc, containing bases − 417 to − 1 of Dsc1 5′-flanking DNA cloned into pGL2-Enhancer, was mutated (by deletion of the 8 bp core sequence) using primer 5 and a primer of complementary sequence.

Multiple copies of the putative Dsc3 C/EBP site at − 193 were generated as follows: an oligonucleotide (primer 6) consisting of two 30 bp repeats flanked by *Sma*I sites was synthesized. Each repeat consisted of the 14 bp C/EBP site sequence with 8 bp 5′- and 8 bp 3′-flanking DNA. Primer 6 was hybridized to a primer of complementary sequence, 5′-phosphorylated and cloned into the *Sma*I site (at position − 290) of plasmid pDsc3(293)luc. DNA sequencing was used to determine the orientation and number of copies of the insert.

Full-length cDNAs encoding rat C/EBPα and C/EBPβ cloned into the expression vector pcDNA3 (Invitrogen) were generously provided by Dr R. C. Smart (North Carolina State University). A clone encoding the truncated C/EBPβ isoform LIP (liver-enriched transcriptional inhibitory protein; amino acids 153–297) [35] was amplified by PCR using primers 7 and 8 (Table 1), sequenced and cloned into pcDNA3. Mouse C/EBPδ cDNA was isolated by reverse transcriptase-mediated PCR. Total RNA was isolated from 2-day-old mouse epidermis by guanidinium isothiocyanate extraction. Before RNA extraction, the epidermis and

dermis were separated using 0.25 % trypsin [36]. RNA was reverse-transcribed using random primers and the first-strand cDNA synthesis kit (Roche). Amplification was then performed by PCR using C/EBPδ-specific primers 9 and 10 (Table 1). Amplified DNA was sequenced and cloned into pcDNA3.

RNase protection assay

RNase protection assays were performed as described in [37]. Total RNA was isolated from 2-day-old mouse epidermis (as above). To produce antisense probes, Dsc3 and Dsc1 5′-flanking DNAs were cloned into Bluescript SK+. Dsc3 and Dsc1 probes were synthesized using T7 polymerase and [α-^{32}P]CTP, and consisted of bases -1 to -596 (*Nco*I) and bases -1 to -536 (*Eco*RI) respectively. Labelled transcripts were hybridized to either mouse epidermal or yeast RNA (20 μg) at 45 °C for 16 h. RNA was digested with RNase A (20 μg/ml) and RNase T1 (250 units/ml) at 25 °C for 30 min. Protected fragments were resolved on 6 % (v/v) denaturing polyacrylamide gels along with DNA sequencing ladders and detected by autoradiography.

Transfection of primary keratinocytes and luciferase assays

Primary mouse keratinocytes were isolated and cultured as described in [36]. Cells were maintained at 34 °C in a low-calcium (0.05 mM) medium and transfected at 80 % confluence (5–10 days after seeding). Transfections were performed in 24-well plates using LIPOFECTAMINE™ reagent (Invitrogen) and serum-free medium following the manufacturer's instructions. Reporter plasmids (Dsc3, pGL3-Basic; Dsc1, pGL2-Enhancer) containing either Dsc3 or Dsc1 5′-flanking DNA upstream of the firefly-luciferase cDNA were used to monitor the promoter activity. In standard Dsc3 and Dsc1 reporter assays, 150 and 500 ng of reporter plasmids respectively were added per well, and transfection efficiency was monitored using plasmid pRL-TK (30 ng), which contains *Renilla* luciferase downstream of the herpes simplex virus thymidine kinase promoter. To determine the effects of C/EBPs on transcriptional activity, plasmids pDsc3(293)luc and pDsc1(417)luc, containing 293 and 417 bp of Dsc3 and Dsc1 5′-flanking DNA respectively, were used. In each case, 150 or 500 ng of the Dsc3 or Dsc1 reporter plasmid was co-transfected with 50 ng of pcDNA3 expression plasmid containing DNA encoding either C/EBPα, C/EBPβ or C/EBPδ. In these experiments, we were unable to monitor transfection efficiency using pRL-TK, since *Renilla* luciferase activity was adversely affected by co-transfection with pcDNA-based plasmids (presumably because of *trans* effects between promoters). Cells from individual mouse skins were routinely divided equally between the wells of 24-well plates. Hence, variations in transfection efficiency between the wells of individual plates were relatively small, and were accounted for by performing multiple repeat experiments (i.e. each transfection experiment was performed at least three times in triplicate). To account for variations in transfection efficiency between plates (the greatest source of potential error), control transfections [either pDsc3(293)luc plus empty pcDNA3 or pDsc1(417)luc plus pcDNA3] were included on each plate. In all experiments, the transfection mixture was removed after 5 h and replaced with standard low-calcium culture medium. Calcium concentration was increased to 0.15 mM after 24 h; the cells were maintained for another 24 h and then lysed in passive lysis buffer (Promega). Firefly and *Renilla* luciferase activities were measured using the Dual-luciferase reporter assay system (Promega). Results are expressed as means $\pm$ S.D.

EMSAs (electrophoretic mobility-shift assays)

Nuclear extracts were prepared as described in [38] from either primary mouse keratinocytes or transfected SV40 (simian virus 40)-transformed African green monkey kidney COS-7 cells. Primary keratinocytes were placed in a normal calcium (0.15 mM) medium for 24 h before harvesting to induce differentiation. COS-7 cells were transfected 48 h before harvesting with C/EBP expression plasmids (3 μg of DNA/9 cm dish) using LIPOFECTAMINE™ 2000 (Invitrogen). EMSAs were performed with 3 μg of nuclear extract and 3×10^4 c.p.m. of end-labelled double-stranded oligonucleotides. Binding reactions were performed at room temperature (22 °C) for 30 min in 20 μl of DNA-binding buffer [20 mM Tris, pH 7.5/1 mM $MgCl_2$/0.5 mM dithiothreitol/0.5 mM EDTA/2 mM spermidine/0.1 mg/ml BSA/5 % (v/v) glycerol]. Poly(dI-dC) (1 μg) was added to each reaction as non-specific DNA. For supershifting assays, 1 μl of antibody was incubated with nuclear extracts before the addition of radiolabelled probe and poly(dI-dC). Rabbit antibodies against C/EBPα, C/EBPβ and C/EBPδ were purchased from Santa Cruz Biotechnology. Protein–DNA complexes were resolved on 6 % polyacrylamide gels. After electrophoresis, gels were dried and exposed to an X-ray film. Oligonucleotides used in EMSAs are listed in Table 1.

Western-blot analysis

Proteins were resolved by SDS/PAGE and transferred on to PVDF membranes (Amersham Biosciences). Blots were probed with rabbit antibodies against C/EBPα, C/EBPβ and C/EBPδ (1:2000; Santa Cruz Biotechnology). Primary antibodies were detected using a peroxidase-conjugated, anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) and the ECL® detection system (Amersham Biosciences).

RESULTS

Characterization of mouse Dsc3 and Dsc1 promoters

5′-Flanking DNA from the mouse *Dsc3* and *Dsc1* genes was isolated from PAC clone 373l6 and λ clone C1 [10] after genomic library screens using cDNA probes. Transcription-initiation sites for mouse Dsc3 and Dsc1 were identified using RNase protection assay (Figure 1A). For Dsc3, two major transcription-initiation sites (at -150 and -102) and a number of minor sites were identified. For Dsc1, there appeared to be one major site of transcription initiation at -252, as well as a number of minor sites (Figure 1A). Analysis of Dsc3 and Dsc1 5′-flanking sequences (GenBank® accession numbers AJ272311 and AF069759) revealed no TATA or CAAT boxes in the vicinity of the transcription start sites.

Dsc3 and Dsc1 upstream sequences were cloned into luciferase reporter gene vectors. A 4 kb fragment of Dsc3 5′-flanking DNA and a deletion series derived from it were cloned into pGL3-Basic, a firefly-luciferase reporter plasmid that lacks eukaryotic promoter and enhancer sequences. These plasmids were transfected into primary mouse epidermal keratinocytes. Surprisingly, the longest fragment (4 kb) gave a relatively low luciferase activity, although this activity was still five times that of the empty vector, indicating that this fragment contains sequences capable of promoter activity (Figure 2). The highest activity was obtained with a 293 bp fragment (four times that of the 4 kb fragment and 20 times that of the empty vector). The difference in activity ($P < 0.001$) between the 293 bp and 4 kb fragments could indicate the presence

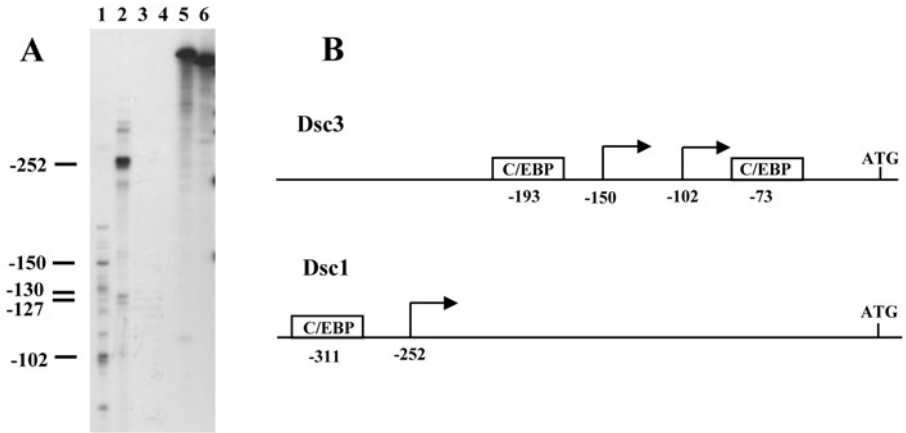

Figure 1 Identification of transcription start sites for mouse Dsc3 and Dsc1

(**A**) RNase protection assay. Antisense probes were hybridized to either mouse epidermal RNA (lanes 1 and 2) or yeast RNA (lanes 3 and 4). Lanes 1 and 3, Dsc3-protected fragments; lanes 2 and 4, Dsc1-protected fragments; lane 5, Dsc3 probe; lane 6, Dsc1 probe. No bands were observed when the Dsc1 antisense probe was hybridized to epidermal RNA from a Dsc1-null mouse [10] (results not shown). The sizes of the fragments were estimated by comparison with a DNA sequencing ladder. (**B**) Dsc3 and Dsc1 5′-flanking DNAs. The positions of the major transcription start sites (arrows), putative C/EBP binding sites and ATG translation start sites are indicated.

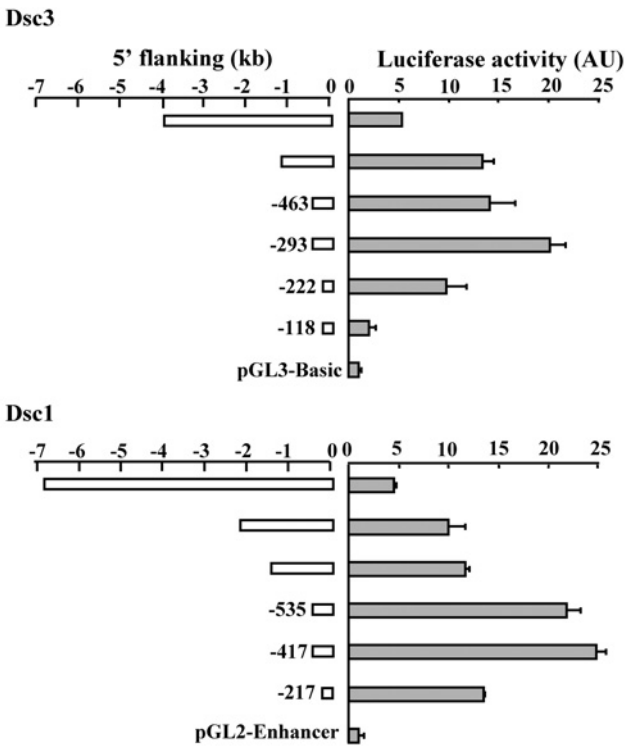

Figure 2 Deletion analysis of Dsc3 and Dsc1 promoter activities in primary mouse keratinocytes

Cells were transiently transfected with Dsc promoter–firefly-luciferase constructs. Dsc3 5′-flanking DNA was cloned into the vector pGL3-Basic, whereas Dsc1 5′-flanking DNA was cloned into pGL2-Enhancer. Constructs were transiently transfected into cultured cells, and firefly-luciferase activity was measured 48 h after transfection. Transfection efficiency was monitored using the plasmid pRL-TK (see the Experimental section). Each bar represents the means ± S.D. for at least three independent transfection experiments performed in triplicate. AU, arbitrary units.

of distal negative regulatory elements. The smallest fragment tested (118 bp) showed activity that was only twice that of the vector (Figure 2). Reversing the orientation of the 293 bp fragment in pGL3-Basic resulted in a 4.9-fold decrease in transcriptional activity (results not shown). A similar pattern of activity was observed when Dsc3 constructs were transfected into primary dermal fibroblasts (results not shown), with the activity of the 293 bp fragment being 17 times that of the empty vector.

A 6.8 kb fragment of Dsc1 5′-flanking sequence and a deletion series derived from it were first cloned into pGL3-Basic. In all cases, low luciferase activity was obtained in cell lysates (results not shown). For this reason, all subsequent experiments on the Dsc1 promoter were performed using the vector pGL2-Enhancer, which contains an SV40 enhancer downstream of the luciferase gene. The presence of the SV40 enhancer resulted in higher levels of luciferase transcription, therefore it may be that enhancers or other genetic elements that are required for high-level Dsc1 transcription are not present in the 6.8 kb fragment. The longest fragment of the Dsc1 promoter gave the lowest luciferase activity (Figure 2), although this activity was still approx. five times greater than that obtained with the empty vector. A 417 bp fragment showed the highest luciferase activity (five times that of the 6.8 kb fragment and 25 times that of the empty vector). A gradual decrease in activity was seen with fragments of intermediate size (535 bp, 1.6 kb and 2.1 kb). The smallest fragment tested (217 bp) was approx. 14 times more active than the empty vector (Figure 2). This fragment lacks the major transcription-initiation site at −252 (Figure 1A): transcription is initiated probably at alternative sites such as those at −127 and −130 (Figure 1A) in this construct. Reversing the orientation of the 417 bp fragment in pGL2-Enhancer resulted in a 3.7-fold decrease in luciferase activity (results not shown). Again, a similar pattern of activity was observed when Dsc1 5′-flanking DNA was transfected into primary dermal fibroblasts (results not shown), with the activity of the 417 bp construct being 25 times that of the empty vector. Thus it appears that the transcription factors necessary to drive *Dsc3* and *Dsc1* gene expressions are present in fibroblasts. It could be that suppression of *Dsc* gene activity in fibroblasts is normally maintained by negative regulatory sequences that are missing in our constructs. Alternatively, regulation may be conferred by tissue-specific differences in chromatin structure in the region of the desmosomal cadherin gene cluster that is not manifested in plasmid DNA.

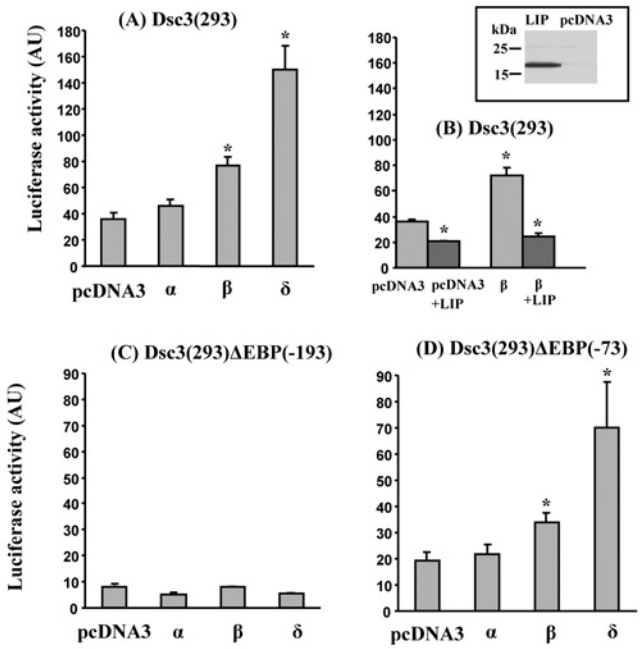

Figure 3 A Dsc3 promoter-luciferase reporter was transactivated by C/EBPβ and C/EBPδ, but not by C/EBPα, and this required the putative C/EBP site at −193 but not that at −73

(**A**) Reporter plasmid pDsc3(293)luc, containing bases −293 to −1 of Dsc3 5′-flanking DNA, was co-transfected into keratinocytes with either empty expression plasmid pcDNA3 or pcDNA3 plus C/EBPα, C/EBPβ or C/EBPδ cDNAs. (**B**) Plasmid pDsc3(293)luc was co-transfected with pcDNA3-LIP (or an equivalent amount of empty pcDNA3) and either pcDNA3 or C/EBPβ expression plasmid. Inset: Western blot of COS-7 cell lysates after transfection with pcDNA3-LIP or empty pcDNA3. The blot was probed with a C/EBPβ-specific antibody. (**C**) Plasmid pDsc3(293)ΔEBP(−193)luc, containing a mutated version of putative C/EBP site at −193, was co-transfected with either pcDNA3, C/EBPα, C/EBPβ or C/EBPδ expression plasmids. (**D**) Plasmid pDsc3(293)ΔEBP(−73)luc, containing a mutated version of putative C/EBP site at −73, was co-transfected with either pcDNA3, C/EBPα, C/EBPβ or C/EBPδ expression plasmids. *$P < 0.001$, significantly different from control by *t* test.

C/EBPs transactivate Dsc promoters

Analysis of Dsc3 and Dsc1 5′-flanking sequences using the MatInspector V2.2 program (http://transfac.gbf.de) revealed many different potential transcription factor-binding sites with a preponderance of AP1, AP2 and AP4 sites. As a first step in identifying the mechanisms that control the expression of Dsc3 and Dsc1, we concentrated our efforts on those fragments of a 5′-flanking DNA that gave the highest activity in keratinocyte transfection experiments, i.e. the 293 bp Dsc3 and 417 bp Dsc1 fragments. Two potential binding sites with high consensus matches were found for C/EBP transcription factors in the proximal Dsc3 promoter (at positions −193 and −73), with one being present in Dsc1 5′-flanking DNA (at position −311; Figure 1B). Interestingly, analysis of 5′-flanking DNA from human *DSC3* (GenBank® accession no. AJ272310) and *DSC1* (Ensembl contig: AC012417.13.1.181552) showed high consensus matches for C/EBP sites in comparable positions for two of these sites (at −210 for *DSC3* and −325 for *DSC1*).

To test whether C/EBPs can regulate Dsc3 transcription, primary mouse keratinocytes were co-transfected with the 293 bp Dsc3 fragment in pGL3-Basic and expression constructs for C/EBPα, C/EBPβ and C/EBPδ (Figure 3A). Expression of C/EBPα had no effect when compared with the empty expression vector pcDNA3. In contrast, both C/EBPβ and C/EBPδ had a significant activating effect, with C/EBPβ causing a 2.1-fold increase in luciferase activity and C/EBPδ causing a 4.2-fold increase in activity.

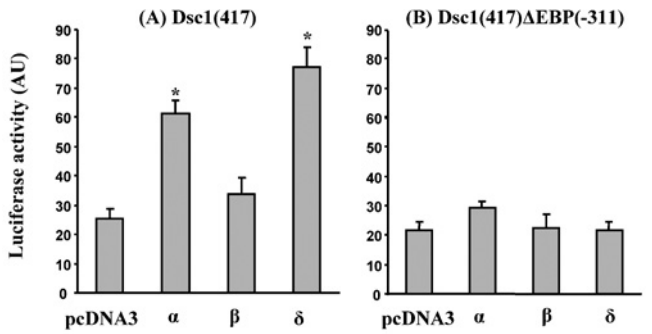

Figure 4 A Dsc1 promoter–luciferase reporter was transactivated by C/EBPα and C/EBPδ, but not by C/EBPβ, and this required the putative C/EBP site at −311

(**A**) Reporter plasmid pDsc1(417)luc, containing bases −417 to −1 of Dsc1 5′-flanking DNA, was co-transfected with either pcDNA3 or C/EBPα, C/EBPβ or C/EBPδ expression plasmids. (**B**) Plasmid pDsc1(417)ΔEBP(−311)luc, containing a mutated version of the putative C/EBP site at −311, was co-transfected with either pcDNA3, C/EBPα, C/EBPβ or C/EBPδ expression plasmids. *$P < 0.001$, significantly different from control by *t* test.

To confirm the role of C/EBPβ in the activation of Dsc3 transcription, further experiments were performed with a clone encoding the C/EBPβ dominant-negative isoform LIP [34]. Transfection of pcDNA3-LIP into COS-7 cells, followed by Western-blot analysis of cell lysates with a C/EBPβ-specific antibody, revealed a strong band of 20 kDa, corresponding to the full-length LIP protein (Figure 3B, inset). Transfection of LIP into keratinocytes resulted in a small decrease in the basal transcriptional activity of the 293 bp Dsc3 reporter construct, probably due to inhibition of endogenous keratinocyte C/EBPs (Figure 3B). Co-transfection of LIP and C/EBPβ abrogated the activating effect of C/EBPβ and resulted in decrease in luciferase activity to below basal levels (Figure 3B).

Further experiments were performed after mutation of the putative C/EBP sites at −193 and −73. We first compared the luciferase activity of mutant (Figures 3C and 3D) and wild-type (Figure 3A) constructs in experiments with pcDNA alone. Mutation of the C/EBP site at −193 caused a 4.4-fold decrease in overall transcriptional activity and abolished the transactivating ability of C/EBPβ and C/EBPδ (Figure 3C). Mutation of the C/EBP site at −73 also resulted in some decrease in the overall activity (1.9-fold), but did not affect the ability of C/EBPβ and C/EBPδ to stimulate luciferase transcription (Figure 3D). These results suggest that the C/EBP site at −193 probably plays an important role in the activation of the Dsc3 promoter by C/EBPs.

To test the role of C/EBPs in Dsc1 transcription, keratinocytes were transfected with plasmid pDsc1(417)luc containing bases −417 to −1 of Dsc1 5′-flanking DNA upstream of the luciferase cDNA. In these experiments, transactivation of the promoter occurred when keratinocytes were co-transfected with C/EBPα and C/EBPδ (2.4- and 3-fold respectively) but not C/EBPβ (Figure 4A). Transactivation by both C/EBPα and C/EBPδ was abolished after mutagenesis of the putative C/EBP site at −311 (Figure 4B).

C/EBPs interact with the Dsc3 and Dsc1 promoters

EMSAs were performed to study transcription factor binding to the Dsc C/EBP sites. Using nuclear extracts from primary mouse keratinocytes, specific complexes were obtained using

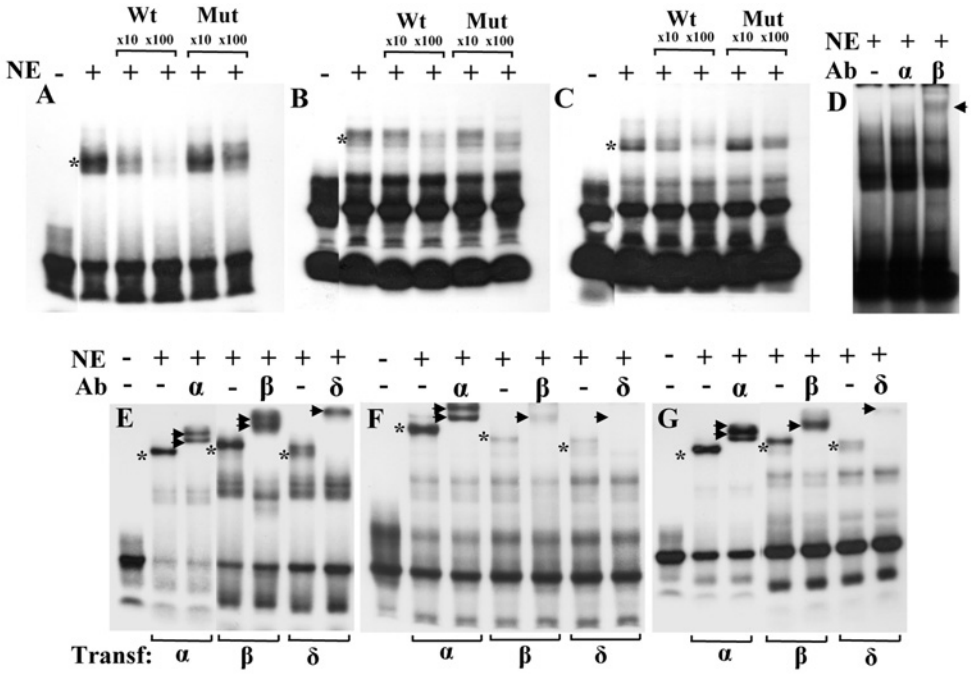

Figure 5 C/EBPα, C/EBPβ and C/EBPδ are capable of interacting with both the Dsc3 C/EBP sites (at −193 and −73) and the Dsc1 C/EBP site at −311

EMSAs with nuclear extracts from primary keratinocytes and (**A**) radiolabelled double-stranded oligonucleotide 11 (corresponding to the C/EBP site at −193 of the Dsc3 promoter), (**B**) radiolabelled double-stranded oligonucleotide 12 (Dsc3 C/EBP at −73) and (**C**) radiolabelled double-stranded oligonucleotide 13 (Dsc1 C/EBP at −311). Specific DNA–protein complexes are indicated by asterisks. Competition experiments were performed with 10- and 100-fold molar excess of unlabelled double-stranded wild-type (Wt) and C/EBP mutant (Mut) oligonucleotides. (**D**) EMSAs with nuclear extracts from primary keratinocytes, oligonucleotide 11 and specific antibodies against C/EBPα and C/EBPβ. A supershifted band (arrowhead) was observed in the presence of the C/EBPβ antibody after prolonged exposure of the X-ray film. EMSAs with nuclear extracts from transfected COS-7 cells and oligonucleotides 11 (**E**), 12 (**F**) and 13 (**G**). In each case, the specific DNA–protein complex (indicated by asterisk) was supershifted in the presence of C/EBP-specific antibodies (arrowheads). In (**F**), a supershifted band was observed with the C/EBPδ antibody after prolonged exposure of the X-ray film. It is not clear why doublets of supershifted bands were obtained with C/EBPα and C/EBPβ antibodies. NE, nuclear extract; Ab, antibody.

radiolabelled double-stranded oligonucleotide 11 (corresponding to the Dsc3 C/EBP site at −193), oligonucleotide 12 (Dsc3 C/EBP site at −73) and oligonucleotide 13 (Dsc1 C/EBP site at −311; Figures 5A–5C). In each case, competition with 10–100-fold molar excess of unlabelled double-stranded wild-type oligonucleotide resulted in the virtual elimination of the shifted band. Competition experiments with C/EBP mutant oligonucleotides had a much decreased effect on the shifted bands obtained for oligonucleotides 11 and 13 (Figures 5A and 5C respectively) when compared with that observed for the wild-type oligonucleotides, although some decrease in the intensity of shifted bands was observed at a 100-fold excess of mutant oligonucleotide, probably as a result of non-specific binding. There was no difference in competition experiments between wild-type oligonucleotide 12 and a C/EBP mutant version (Figure 5B). This suggests that the shifted bands in the case of oligonucleotides 11 and 13 could be due to the binding of C/EBPs, whereas that obtained with oligonucleotide 12 may be the result of other, unknown factors binding to the probe.

To obtain further evidence for the regulation of the Dsc3 promoter by C/EBPs, we performed supershifting analysis with keratinocyte nuclear extracts and specific antibodies against C/EBPα and C/EBPβ. C/EBPδ was not included in this analysis since it is expressed at extremely low levels in cultured primary mouse keratinocytes (see below). Using a specific antibody against C/EBPβ, we were able to supershift the complex obtained with oligonucleotide 11 (Dsc3 C/EBP site at −193; Figure 5D). However, despite repeated attempts, we could not demonstrate the presence of a supershifted band with an antibody against C/EBPα. These results support the idea that the Dsc3 promoter

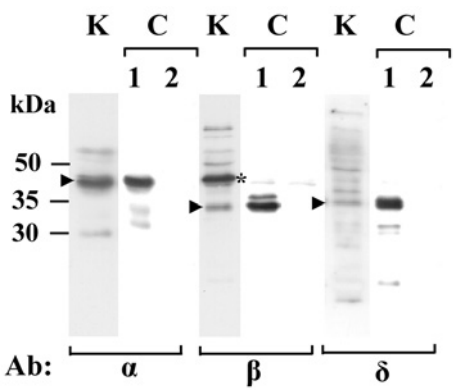

Figure 6 Expression of C/EBPs in nuclear extracts from primary keratinocytes and transfected COS-7 cells

Western blot of lysates from cultured keratinocytes (non-transfected) and COS-7 cells transfected with cDNAs encoding either C/EBPα, C/EBPβ or C/EBPδ cDNAs (in the expression vector pcDNA3) or empty pcDNA3. Blots were probed with C/EBPα-, C/EBPβ- or C/EBPδ-specific antibodies, and full-length proteins are indicated by arrowheads. C/EBPs are expressed at low levels in keratinocytes and were detected only after prolonged exposure of the X-ray film to ECL®-generated luminescence. The band indicated by an asterisk is non-specific. K, keratinocyte; C, COS-7 cell transfected with C/EBP cDNA (lane 1) or empty pcDNA3 (lane 2).

is differentially regulated by C/EBPs (see Figure 3A). However, the results should be treated with some caution. From Western-blot analysis, it was found that all three C/EBPs (but especially C/EBPδ) are present at very low levels in nuclear extracts from primary keratinocytes (Figure 6). We therefore prepared nuclear

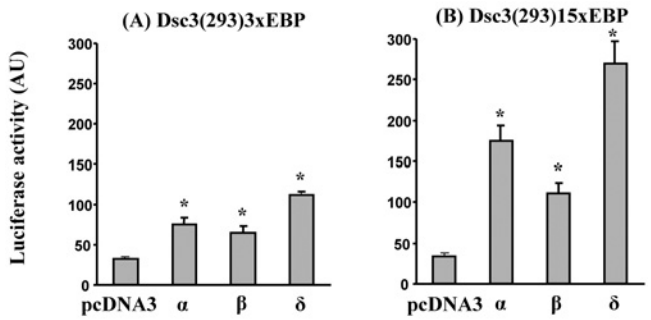

Figure 7 The relative transactivating abilities of C/EBPs were altered by multimerization of the Dsc3 site at −193

(**A**) Plasmid pDsc3(293)3 × EBPluc, containing two additional copies of the C/EBP site at −193, was co-transfected with either pcDNA3 or C/EBPα, C/EBPβ or C/EBPδ expression plasmids. (**B**) Plasmid pDsc3(293)15 × EBPluc, containing 14 additional copies of the C/EBP site at −193, was co-transfected with either pcDNA3 or C/EBPα, C/EBPβ or C/EBPδ expression plasmids. *$P < 0.001$, significantly different from control by *t* test.

extracts from COS-7 cells transfected with cDNAs encoding either C/EBPα, C/EBPβ or C/EBPδ. Western-blot analysis of both cell lysates and nuclear extracts from transfected COS-7 cells with C/EBP-specific antibodies revealed strong bands of 42, 35 and 34 kDa, corresponding to full-length C/EBPα, C/EBPβ and C/EBPδ proteins respectively (Figure 6). Using nuclear extracts from transfected COS-7 cells, shifted bands were obtained with oligonucleotides 11, 12 and 13 by EMSA (Figures 5E–5G). Furthermore, antibodies against all three C/EBPs caused supershifts in the shifted bands (Figures 5E–5G). These results show that in the presence of excess C/EBPs, all three transcription factors are capable of interacting with both Dsc3 and Dsc1 C/EBP sites. Hence, the possibility exists that the absence of a supershifted band with the C/EBPα antibody (Figure 5D) is due to low levels of C/EBPα in nuclear extracts prepared from cultured keratinocytes. The results shown in Figure 5 also demonstrate that the apparent lack of participation of the Dsc3 C/EBP site at −73 in the regulation of the *Dsc3* gene (Figure 3D) cannot be explained by the inability of C/EBPs to bind to the site. To eliminate the possibility that C/EBPs could interact with the mutated site at −73, we annealed and labelled oligonucleotides 3 and 4 (used to generate the mutant site) and performed EMSAs as before. No significant binding of C/EBPs to the mutant site was found (results not shown).

Multimerization of the Dsc3 C/EBP site at −193 alters the relative transactivating abilities of the C/EBPs

To address the possibility that the environment of a particular C/EBP site could affect the binding of C/EBPs, we manipulated the environment of the Dsc3 site at −193 by inserting multiple copies of the site (14 bp core sequence plus 8 bp 5′- and 8 bp 3′-flanking DNA) into an *Sma*I site (at −290) of plasmid pDsc3(293)luc. In the presence of two more copies, C/EBPα could activate luciferase transcription (2.4 times greater than basal levels; Figure 7A), although it had no effect on the original, parental construct (Figure 3A). In contrast, the transactivating abilities of C/EBPβ and C/EBPδ were not affected. When 14 additional copies of the site (all in the same orientation) were cloned into the plasmid pDsc3(293)luc, the transactivating ability of C/EBPα was again increased (5.3 times greater than basal levels; Figure 7B). The presence of 14 additional copies of the site resulted in a 1.8-fold increase (when compared with the original parental construct; $P < 0.01$) in the transactivating ability of C/EBPδ, but had no significant effect on the ability of C/EBPβ to stimulate luciferase transcription. Hence, the presence of additional C/EBP sites in the 15 × EBP construct clearly alters the relative transactivating abilities of the C/EBPs when compared with the wild-type construct (cf. Figure 7B with 3A). In the 15 × EBP construct, C/EBPα is more effective at stimulating luciferase transcription compared with C/EBPβ, although it remains less effective than C/EBPδ.

DISCUSSION

An important goal for epidermal cell biology is to identify transcription factors that are responsible for the stratum-specific expression of genes during keratinocyte differentiation. In the present study, we have identified key regulatory elements in the promoters of two *Dsc* genes (*Dsc3* and *Dsc1*) and showed that transcription factors of the C/EBP family regulate the activity of both. This is the first study that has identified transcription factors involved in the regulation of desmosomal cadherin genes, although a number of studies have identified minimal promoter regions from *Dsc* and *Dsg* genes that direct the activity of reporters to epithelial cells in culture [39–42].

Dscs are expressed in a differentiation-specific manner during keratinocyte differentiation. In bovine tissues, Dsc3 and Dsc1 show graded distributions, with Dsc3 expression decreasing towards the upper suprabasal cell layers and Dsc1 showing the reciprocal pattern [17]. C/EBPs are also expressed in a differentiation-specific manner. Thus C/EBPβ is abundant in the lower/middle epidermis, whereas C/EBPα is found largely in suprabasal cell layers [31,33]. C/EBPδ appears to be expressed in all layers of interfollicular epidermis [32]. Our results are consistent with a model wherein C/EBPβ and C/EBPδ enhance Dsc3 expression and may in part explain why Dsc3 is most strongly expressed in lower cell layers, where C/EPBβ predominates. In contrast, C/EBPα and C/EBPδ appear to up-regulate Dsc1 expression, which again may explain in part why Dsc1 expression is strongest in upper, suprabasal layers of the epidermis where C/EBPα predominates.

In reality, regulation of *Dsc* gene expression is probably more complex and may involve competition between different C/EBPs for binding at Dsc promoters. Alternatively, the environment of a particular site may favour the binding of one C/EBP over that of another. This idea is supported by our results, which show that all three C/EBPs are capable of interacting with Dsc3 and Dsc1 sites (Figure 5), but only C/EBPβ and C/EBPδ activate Dsc3 transcription (Figure 3), whereas Dsc1 activity is enhanced by C/EBPα and C/EBPδ alone (Figure 4). Furthermore, the relative transactivating abilities of the C/EBPs can be manipulated by altering the context of a site. Thus multimerization of the Dsc3 C/EBP site (at −193) has no effect on the transactivating ability of C/EBPβ, but does significantly enhance the transactivation potential of C/EBPα (Figure 7).

There is considerable potential for additional regulatory interactions. C/EBPα and C/EBPβ are encoded by intronless genes; however, in each case, a single mRNA can produce several protein isoforms. Thus C/EBPβ can produce at least three isoforms, a full-length protein (38 kDa), LAP (liver-enriched activating protein; 35 kDa) and LIP (20 kDa). The mechanisms for the regulation of these isoforms are not well understood, but LIP is proposed to function as a dominant-negative inhibitor of full-length C/EBPs by forming non-functional heterodimers with other family members [35]. C/EBPζ, which is expressed in the epidermis, can also act as a dominant-negative inhibitor of

transcriptional activation by preventing the binding of C/EBPα and C/EBPβ to DNA [43]. Thus LIP and C/EBPζ might counteract the stimulatory effects of activator C/EBP isoforms. Furthermore, C/EBPs have the ability to participate in auto- and cross-regulation [44,45], thereby adding to the potential complexity of desmosomal cadherin gene regulation.

Our results suggest that a gradient of C/EBPs, together with differential regulation of promoters, could be responsible for establishing the differentiation-specific expression patterns exhibited by the Dscs in the epidermis. Such a mechanism may be of more general applicability. Previous studies have shown that C/EBPα up-regulates involucrin, which is first expressed in the upper spinous layer, whereas C/EBPβ and C/EBPδ have an inhibitory effect [34]. Keratin 10, an early marker of differentiation, is activated by both C/EBPα and C/EBPβ, with C/EBPα having a greater effect than C/EBPβ [33]. In contrast, α2 and α5 integrins, whose expression is restricted to the basal cell layer, are repressed by C/EBPα and C/EBPβ, with C/EBPα being the more effective repressor in both cases [46]. Thus, in the latter case, up-regulation of the C/EBPs could contribute to the inhibition of integrin transcription during keratinocyte terminal differentiation. The role of C/EBPs in achieving differentiation-specific expression of other desmosomal cadherins such as Dsgs, which exhibit patterns of expression similar to that of Dscs in the epidermis, and other unrelated genes, needs further investigation.

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