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Characterisation of a desmocollin isoform (bovine DSC3) exclusively expressed in lower layers of stratified epithelia

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SUMMARY

Desmocollins are cadherin-like glycoproteins involved in cell adhesion and plaque formation in desmosome junctions. Three distinct isoforms, the products of different genes, have been found in bovine tissues. We have reported previously that one of these, DSC3, is expressed only in basal and lower suprabasal layers of stratified epithelia. Using RT-PCR we have now obtained the complete cDNA coding sequence of mature bovine DSC3. It has alternatively spliced 'a' and 'b' forms found in other desmocollins but is unique in having a 43 instead of a 46 base pair exon. We have characterised a monoclonal antibody, 07-4G, which is specific for the Dsc3 protein, recognising an epitope in the extracellular domain. Immunofluorescent staining with 07-4G confirms that this isoform is found only in stratified epithelia, being strongly expressed in the basal cell layers of these tissues. The intensity of expression fades gradually in the suprabasal layers and disappears completely below the upper limit of desmosome expression. These results suggest that Dsc3 plays an important role in cell epithelial differentiation.

Key words: desmosome, desmocollin, RT-PCR, expression, stratified epithelium

INTRODUCTION

Desmosomes are punctate adhesive intercellular junctions that associate with the intermediate filament network of the cytoskeleton and are found in epithelia, cardiac muscle, meninges and follicular dendritic cells. They provide strong adhesion sites which resist mechanical stress by conferring structural continuity between cells (for reviews see Garrod, 1993; Garrod and Collins, 1992; Legan et al., 1992; Buxton and Magee, 1992; Magee and Buxton, 1992; Amagai et al., 1994; Koch and Franke, 1994).

The major desomosomal glycoproteins, desmocollins and desmogleins, are both members of the cadherin family of calcium-dependent adhesion molecules (Holton et al., 1990; Koch et al., 1991). Cadherins homology is strongest in the extracellular domains, while the cytoplasmic domains have unique features (Koch et al., 1991; Collins et al., 1991; Mechanic et al., 1991; Parker et al., 1991; Wheeler et al., 1991). Desmocollins, the subject of this paper, show size heterogeneity of their cytoplasmic domains, generated by alternative mRNA splicing (Parrish et al., 1990; Collins et al., 1991; Parker et al., 1991). The longer alternatively spliced form is designated 'a' and the shorter 'b' (Buxton et al., 1993).

Three desmocollin isoforms, the products of different genes (DSC1, 2 and 3), have been cloned from bovine tissues (Collins et al., 1991; Mechanic et al., 1991, Koch et al., 1992; Garrod, 1993; Buxton et al., 1993; Theis et al., 1993; Troyanovsky et al., 1993; Legan et al., 1994). In addition two isoforms have been cloned from human tissues (Parker et al., 1991; Theis et al., 1993; King et al., 1993) and one from mouse (King et al., 1993; Lorimer et al., 1994). Recently, a cDNA called HT-CP was cloned from a human bladder carcinoma library (Kawamura et al., 1994) in which the sequence has extensive homology with the BDSC3 partial sequence published by Legan et al. (1994). All desmocollins described so far have alternatively spliced a and b forms.

Expression of the desmocollin isoforms correlates strongly with epithelial differentiation (Arnemann et al., 1993; Theis et al., 1993; Legan et al., 1994; Lorimer et al., 1994). Legan et al. (1994) have shown that Dsc1 is strongly expressed in epidermis and papillae of tongue epithelium but at only very low intensity elsewhere. Dsc2 is ubiquitously expressed in epithelia and cardiac muscle, while Dsc3 expression appears restricted to stratified and tracheal epithelia. In situ hybridisation studies in stratified epithelia indicate that Dsc1 is associated with 'skin'-type terminal differentiation in epidermis and tongue, Dsc2 is most strongly associated with the immediately suprabasal layers, while Dsc3 is expressed at highest intensity in basal layers (Legan et al., 1994).

This paper further characterises DSC3, which was previ-

ously known only from partial sequence (Theis et al., 1993; Legan et al., 1994), and confirms its restricted expression in stratified epithelia. Using RT-PCR we have obtained the entire cDNA sequence of bovine DSC3. In this paper we also describe a monoclonal antibody, 07-4G, and use it to investigate the pattern of Dsc3 protein expression in bovine stratified epithelia. Dsc3 expression is strong basally and decreases gradually in the suprabasal layers. This distribution suggests that Dsc3 is specifically associated with proliferative and early differentiative processes in stratified epithelia.

MATERIALS AND METHODS

Isolation of total RNA

Tissue (2 g) from bovine nasal epidermis was dissected, frozen immediately in liquid nitrogen and pulverized with a mortar and pestle cooled with liquid nitrogen. Total RNA was isolated according to the method of Chomczynski and Sacchi (1987).

Reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

Randomly-primed first strand cDNA synthesis was carried out from total RNA (1 μg) using a first strand cDNA synthesis kit (Amersham International). An aliquot (1 μl) of the first strand cDNA reaction was then added directly to a PCR mixture containing 0.2 mM of each dNTP, sense and anti-sense PCR primers (listed below), 1.5 mM $MgCl_2$ and 2.5 U Taq DNA polymerase in 100 μl (1×) DNA polymerase buffer (50 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100). The whole reaction mixture was overlaid with mineral oil (Sigma).

PCR primers (with internal restriction sites underlined) were as follows: KS3, GGGAATTCGA(T/C)GA(A/G)GGNCCNGA-(A/G)TG (*Eco*RI), 128-fold redundant, 640 pmol/reaction; YA3, GCGAATTCTTATTTCTGGCACTAAC (*Eco*RI), 50 pmol/reaction; YS6, GGCGAATTCTGGGCNCCNAT(T/C/A)CCNTG (*Eco*RI), 192-fold redundant, 960 pmol/reaction; YA5, CATGGGAATTC-TAGCCACTGATCTTTGA (*Eco*RI), 100 pmol/reaction; YS8, GGGATCCAGATGGGCGCCGATACCC (*Bam*HI), 100 pmol/reaction; YA6, GGGATCCTTCAAAATTATAAACTGC (*Bam*HI), 100 pmol/reaction; YS7, GGCGGATCCGAATTGTTAGTGCCA-GAAAT (*Bam*HI), 100 pmol/reaction; YA10, GCGGATCCGT-GATGGCATGTGCTCTT (*Bam*HI), 100 pmol/reaction; YS20, ACAACGCTAACCAGGGCTTTTGTGGC, 100 pmol/reaction; YA23, CCCGGGTTANC(T/G)(T/C)TTNGT(A/G)CANGT(T/C)TT (*Sma*I), 1024-fold redundant, 5120 pmol/reaction. A Techne PHC-3 Thermal Cycler was used with the following cycling times and temperatures: KS3/YA3, YS8/YA6 and YS20/YA23, 94°C 2 minutes, followed by 5 cycles of 50°C 1 minute, 72°C 1 minute, 94°C 45 seconds, and 30 cycles of 65°C 1 minute, 72°C 1 minute, 94°C 45 seconds, then 72°C 5 minutes; PCR using YS6/YA5 and YS7/YA10, the same cycling times as KS3 and YA3 except the extension time (at 72°C) was 75 seconds. Reaction products were analysed by agarose gel electrophoresis and visualised by staining with ethidium bromide.

Isolation of 5′ sequence by rapid amplification of cDNA ends (5′ RACE)

5′ Sequence encoding the N terminus of mature Dsc3, as well as part of the prosequence, was obtained using the 5′ RACE kit from Gibco/BRL. Two nested antisense Dsc3-specific primers (YA6 and YA7) were made to the 5′ region of the known sequence. The sequence of YA6 is listed above. YA7, GGGATCCAATCAGAT-CAAAAACGTC (*Bam*HI), 100 pmol/reaction; Anchor primer, CUACUACUACUAGGCCACGCGTCGACTATACGGGIIGGGII-GGGIIG (Gibco/BRL), 100 pmol/reaction. The Techne Thermal Cycler was used for PCR reactions containing the anchor primer and YA6 with the same cycling times and temperatures as KS3 and YS3. An aliquot (2 μl) of the PCR reaction product was then amplified using the anchor primer and YA7, with the same cycling times and temperatures as before.

DNA sequencing and sequence analysis

Primers were removed from the PCR reaction mixtures using the Magic™ PCR DNA purification system (Promega) and the PCR reaction products were subcloned into pGEM-T vector (Promega). Transformants were screened for the correct insert using PCR screening, in which bacteria from each colony were added to 20 μl of PCR mixture described above. PCR cycling times were as for KS3/YA3 except the first denaturation time was 5 minutes, and in subsequent cycles the denaturation time was 90 seconds. Plasmid DNA was prepared from at least four separate transformants using Magic™ Miniprep columns (Promega). Plasmid DNA was then denatured and sequenced (Sanger et al., 1977; Sambrook et al., 1989) in both directions using T7 and SP6 primers (Promega) and Sequenase V2.0 (USB). In order to obtain sequence of the 780 bp and 1.1 kb clones in both directions, sequencing using internal sequencing primers (SS1,SA1 and SS2,SA2, respectively) derived from known sequences of the clones was also carried out. SS1, AGATGGTAGATCATG-TACT; SA1, CATTAACTCTGGTGATCGT; SS2, AACTGCA-GACGGATATTCA; SA2, TCTGTAGTGATTTTGAAATG. Sequence analysis was carried out using PC-Gene (Intelligenetics Inc.).

Construction of expression plasmids

Construct pGEX3X/Dsc1b, encoding bovine Dsc1b, was made as follows. Clone CN35 (Collins et al., 1991) was cut with *Xba*I, blunt-ended with Klenow fragment and ligated into pGEX3X (Pharmacia) cut with *Sma*I.

Construct pGEX4T/Dsc2b, encoding bovine Dsc2b, was made as follows. Clone BMDCT2-DC4.1 (Koch et al., 1992) was cut with *Sac*I, blunt-ended with Klenow fragment and cut with *Xho*I. The 3.0 kb DNA fragment was isolated and subcloned into pGEX4T-3 vector (Pharmacia) cut with *Sma*I and *Xho*I.

Four expression constructs encoding the complete mature Dsc3b protein (pGEX2T/Dsc3b), the extracellular domain of Dsc3 (pGEX2T/Dsc3-Ec), the cytoplasmic domains of the a form (incomplete) (pGEX2T/Dsc3a-Cyt) and the b form (pGEX2T/Dsc3b-Cyt) were also made.

Plasmid pGEX2T/Dsc3b was made as follows. The DNA fragment (250 bp) generated by RT-PCR using YS1/YA2 (Legan et al., 1994) was digested with *Bam*HI and *Xho*II, while that generated using KS2/A2 (372 bp) was digested with *Xho*II and EcoRI. The fragments were then isolated, ligated together and subcloned into BluescriptII SK^+ (Stratagene) to create a construct which encodes the transmembrane and cytoplasmic domains of Dsc3b. To create a construct which encodes the extracellular and transmembrane domains of Dsc3, the DNA fragment generated by RT-PCR using YS8/YA5 (1.0 kb) was cut with *Bam*HI and *Bsm*I, while that generated using KS3/YA3 (760 bp) was digested with *Bsm*I and *Eco*RI. The fragments were then isolated, ligated together and subcloned into BluescriptII SK^+. The above two constructs were then digested with *Bam*HI and *Bst*XI (partial), the 1.7 kb and 584 bp DNA fragments were isolated, ligated together and subcloned into BluescriptII SK^+ to form a construct which encodes the whole mature Dsc3b protein. This was then digested with *Bam*HI (YS8 and YA2 contain *Bam*HI restriction sites) and the Dsc3b coding sequence subcloned into pGEX2T vector (Pharmacia) cut with *Bam*HI.

Construct pGEX2T/Dsc3-Ec was made by digesting with *Bam*HI and *Eco*RI (partial) the BluescriptII construct containing DNA generated by RT-PCR using YS8/YA5 and KS3/YA3 (described above). Sequence encoding the Dsc3 extracellular domain was then subcloned into pGEX2T vector cut with *Bam*HI and *Eco*RI.

Plasmids pGEX2T/Dsc3b-Cyt and pGEX2T/Dsc3a-Cyt were made by digesting with *Bam*HI the two PCR products (504 bp and 547 bp encoding the cytoplasmic domains of the a and b forms, respectively) generated using YS8 and YA2. These were subcloned into pGEX2T vector which had been cut with *Bam*HI.

Expression and detection of fusion proteins

The plasmids described above, and the vector pGEX2T alone (as a control), were transformed into *E. coli* XL1-blue cells (Stratagene). Transformants were grown for 3 hours in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of the fusion proteins. Cells were harvested, lysed in Laemmli's sample buffer and boiled for 5 minutes. Proteins were then separated by 8% SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose. Blots were blocked in 5% skimmed milk and probed with mouse monoclonal antibodies (52-3D, 07-4G, 07-4D) raised against bovine nasal epidermal desmosomes (Holton et al., 1990).

52-3D is an anti-desmocollin monoclonal antibody previously shown to react with the cytoplasmic domain and have wide tissue cross-reactivity (Parrish et al., 1990; Collins et al., 1991; Garrod and Collins, 1992). 07-4D reacts with the extracellular domain of Dsc1 (Holton et al., 1990; Legan et al., 1994). 07-4G is characterised below. The monoclonal antibodies were applied to blots as tissue culture supernatants and detected with biotinylated anti-mouse IgG (1:15,000) followed by ABC-AP reagent (Vector Laboratories). Colour was developed using BCIP/NBT substrate (Vector Laboratories).

Detection of the desmocollins from desmosome preparations

Desmosomal cores were prepared according to Gorbsky and Steinberg (1981). The proteins were separated by 10% SDS-PAGE and blotted as described previously. The blots were then probed with monoclonal antibodies which were detected with anti-mouse IgG alkaline phosphatase conjugate (1:30,000) (Sigma) and the colour was again developed using the BCIP/NBT substrate.

Immunofluorescence staining

Immunofluorescent staining of bovine tissues (oesophagus, rumen, tongue and nasal epidermis) with monoclonal antibodies 07-4G (described here) and 11-5F, to desmoplakins (Parrish et al., 1987), was detected using a biotinylated rabbit anti-mouse IgG secondary antibody and ExtrAvidin-FITC conjugate (1:50) (Sigma). Antibodies were applied to frozen tissue sections of 7 μm thickness and coverslips were mounted in Vectashield Mounting Medium (Vector Laboratories). Sections were examined using a Zeiss Axioplan microscope.

RESULTS

Bovine Dsc3 sequence

We have previously identified a third bovine desmocollin gene, DSC3, and obtained the cDNA sequence of its transmembrane domain and most of its cytoplasmic domain (Legan et al., 1994). To obtain further 5′ cDNA sequence, RT-PCR was carried out using bovine DSC3-specific non-redundant antisense primers, derived from known DSC3 sequence, and redundant sense primers, derived through comparison of known desmocollin sequences (bovine DSC1 (Collins et al., 1991), DSC2 (Koch et al., 1992) and human DSC2 (Parker et al., 1991)).

Using a redundant sense primer, KS3, from the extracellular domain and a DSC3-specific antisense primer, YA3, immediately downstream of the transmembrane domain (Fig. 1A), a 778 bp PCR product was generated from bovine nasal epidermis total RNA (Fig. 1B, lane 2). After subcloning into pGEM-T vector, four independent clones were checked for the correct insert and sequenced. This extended the sequence in a 5′ direction to position 1084 (Fig. 2).

A new DSC3-specific antisense primer, YA5, was derived from the 5′ end of the new sequence and was used in combination with a redundant sense primer, YS6, corresponding to an extensively conserved protein sequence, WAPIP, at the N terminus of mature desmocollins (Fig. 1A). A 1093 bp PCR product was amplified from bovine total RNA (Fig. 1B, lane 4). Four independent clones were again selected after the PCR product was subcloned into the pGEM-T vector, and subsequently sequenced. This gave the remaining sequence of the extracellular domain of DSC3 except that encoding the first amino acid of the mature protein.

To extend the sequence to that encoding the mature N terminus and the precursor sequence, 5′ RACE (Rapid amplification of cDNA ends) was carried out (Fig. 1A). Five inde-

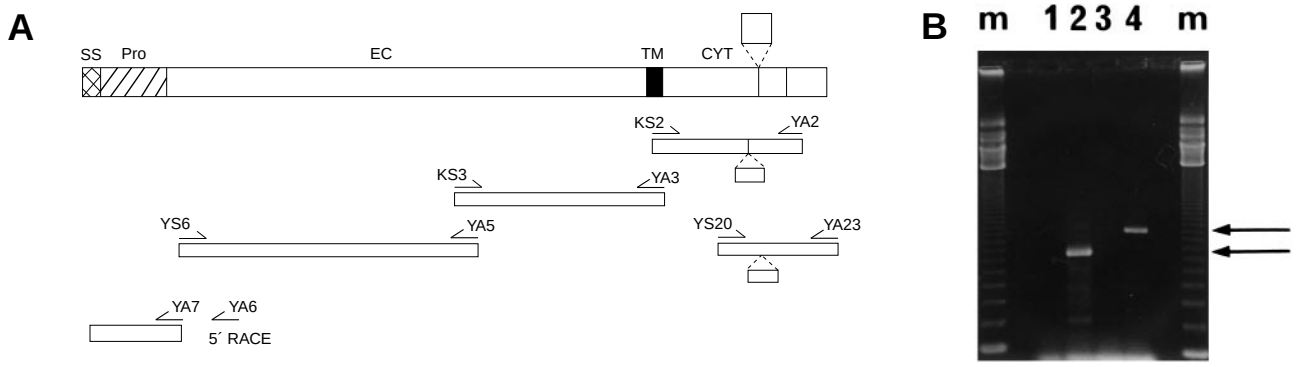

Fig. 1. (A) Strategy for obtaining sequence of DSC3. Schematic diagram showing structure of Dsc3 (SS- signal sequence, Pro- prosequence, EC- extracellular domain, TM- transmembrane domain, CYT- cytoplasmic domain), the PCR primers and their respective PCR reaction products. The proximal part of CYT is common to both a and b forms of Dsc3. This is followed by an alternative splice site which results in C-termini of different lengths. The extreme 5′ end of the coding region has not been obtained (dotted lines). (B) Agarose gel analysis of PCR reaction products. Lanes m are 123 bp markers; lanes 1 and 3, control RT-PCR reactions without total RNA; lanes 1 and 2, RT-PCR reactions primed with KS3/YA3; lanes 3 and 4, RT-PCR reactions primed with YA6/YA5. Specific PCR products of 778 bp and 1093 bp are arrowed.

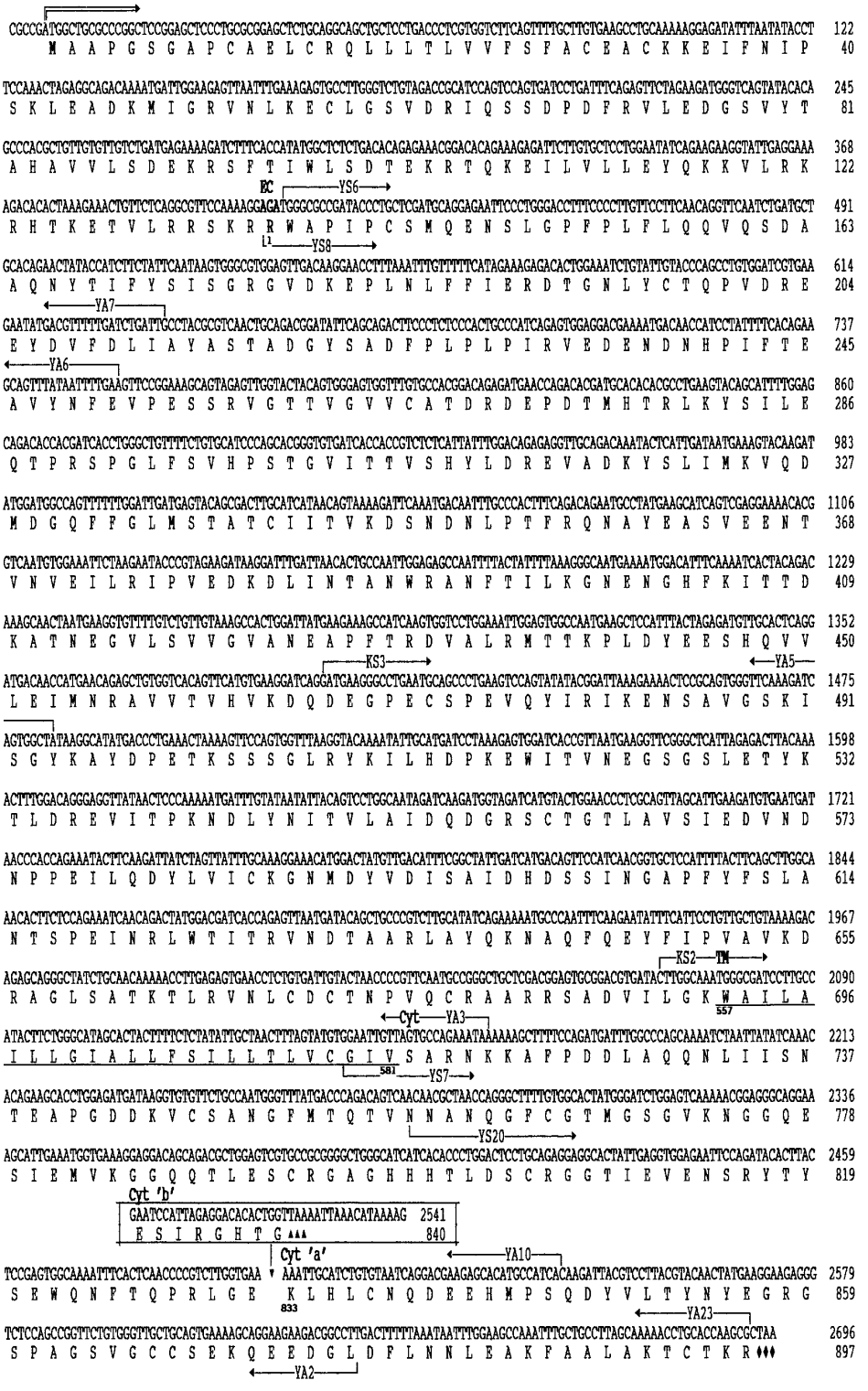

Fig. 2. Sequence of bovine type 3 desmocollin. The nucleotide sequence of DSC3 and its deduced amino acid sequence are shown. The start of the open reading frame is marked with ⌐. The first amino acid of the mature protein is designated as amino acid number 1 (indicated). The predicted transmembrane domain is underlined. The splice site in the cytoplasmic domain is marked with ▼ and the 43 bp exon encoding the C terminus of the b splice variant is boxed. The in-frame stop codon in the exon is marked with ▲▲▲ whereas the stop codon for the 'a' protein is marked with ◆◆◆. The positions of the PCR primers are marked (see Fig. 1). The sequence is available from EMBL/GenBank/DDBJ under accession number L33774.

pendent clones of variable lengths (650-700 bp) were screened and subsequently sequenced. All five contained the cDNA sequence encoding the signal sequence and presequence of BDSC3 (Fig. 2).

The bovine DSC3 sequence identified previously has a 43 bp exon which encodes 8 amino acids followed by a stop codon (Legan et al., 1994). However, all the other known desmocollins contain an exon of 46 bp in length encoding 11 amino acids followed by a stop codon. To verify the DSC3 exon sequence, RT-PCR using DSC3-specific sense and antisense primers (YS7 and YA10) was carried out. Two PCR products of 315 bp and 358 bp were amplified and sequenced. They were found to correspond to the cytoplasmic region of a and b forms of DSC3, respectively, in which the b form contained

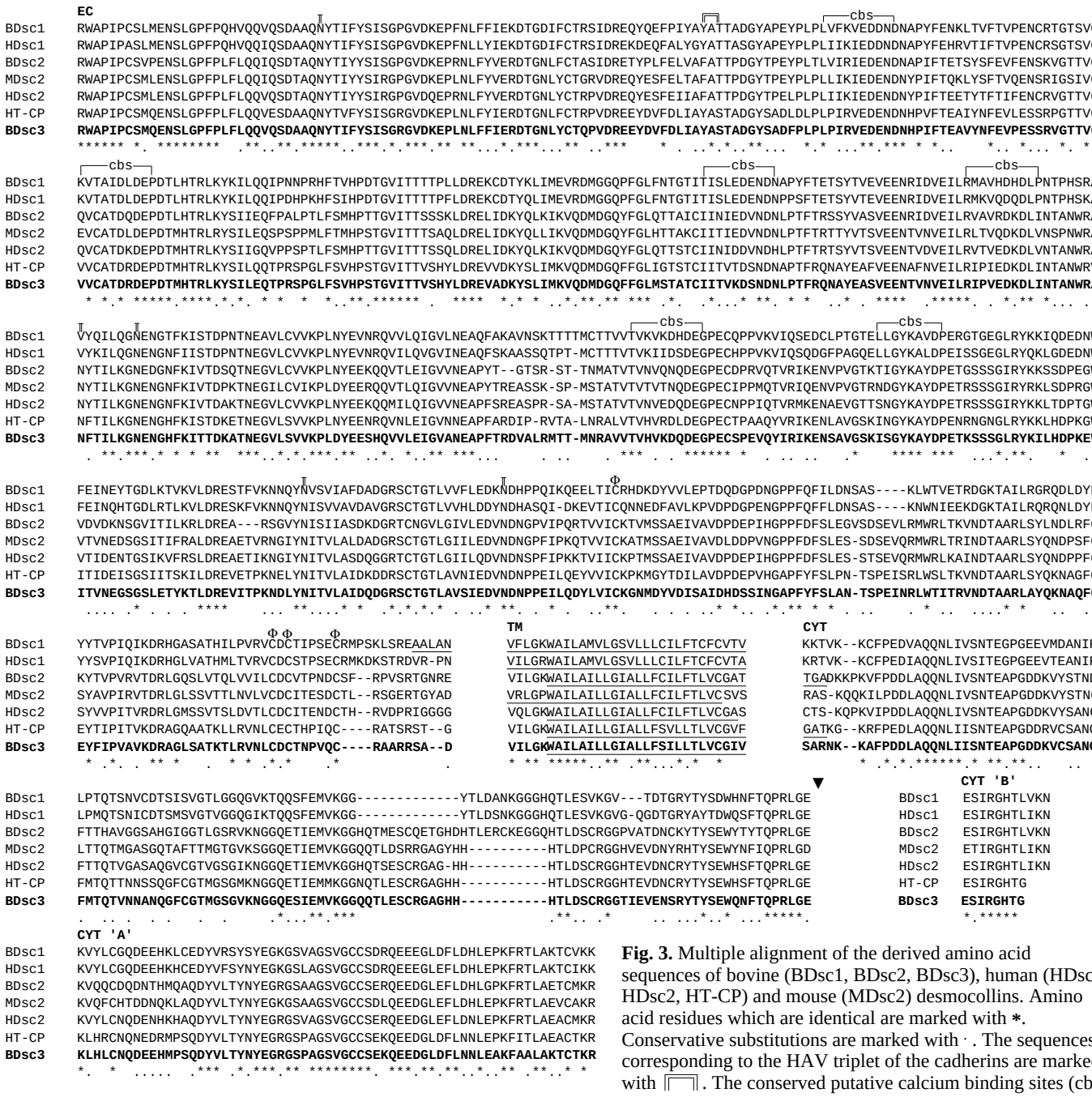

Fig. 3. Multiple alignment of the derived amino acid sequences of bovine (BDsc1, BDsc2, BDsc3), human (HDsc1, HDsc2, HT-CP) and mouse (MDsc2) desmocollins. Amino acid residues which are identical are marked with *. Conservative substitutions are marked with · . The sequences corresponding to the HAV triplet of the cadherins are marked with ⌈⌉. The conserved putative calcium binding sites (cbs) are indicated. The putative N-glycosylation sites are marked with ‖. The conserved cysteine residues adjacent to the transmembrane domain are marked with Φ. The predicted transmembrane domains are underlined (according to that in the original publications) and the cytoplasmic domains after the splice site are shown separately as a and b forms.

once again an exon of 43 bp in length, confirming the sequence obtained by Legan et al. (1994).

To obtain the remaining 3′ end of the bovine DSC3 sequence, RT-PCR using YA23, a redundant antisense primer derived from the human HT-CP sequence (Kawamura et al., 1994) and the BDsc3 sequence published by Troyanovsky et al. (1993), and YS20, a bovine DSC3-specific sense primer, was carried out. Two PCR products of 428 bp and 471 bp were amplified, and five independent clones for each of the two PCR products were sequenced. This completes the coding sequence of the 3′ end of the bovine DSC3.

The bovine DSC3 sequence we have obtained encodes a mature desmocollin protein (Dsc3a) of 762 amino acids with a deduced molecular mass of 84,504 (Fig. 2). This consists of an extracellular domain of 556 amino acids, a hydrophobic region of 25 amino acids which constitutes the transmembrane domain, and a cytoplasmic domain of 181 amino acids (Fig. 2, CYTa). The cDNA sequence which encodes the Dsc3b protein

contains an extra 43 bp exon with an internal stop codon. This results in a shorter cytoplasmic domain of 94 amino acids (Fig. 2, CYTb).

Amino acid sequence comparison

Alignment of the protein sequences of all the known desmocollins (bovine Dsc1 (Collins et al., 1991), Dsc2 (Koch et al., 1992) and Dsc3; mouse Dsc2 (Lorimer et al., 1994); human Dsc1 (Theis et al., 1993), Dsc2 (Parker et al., 1991) and HT-CP (Kawamura et al., 1994)) reveals that the desmocollins show substantial conservation (Fig. 3). The sequence homology is especially high in the N-terminal region of the extracellular domain of the mature proteins and decreases gradually towards the distal portion of the extracellular domain (Fig. 3, EC). Also they all contain four conserved cysteine residues in the extracellular membrane-proximal region (Fig. 3, marked with Φ). All contain six putative calcium-binding sites, some of which are highly conserved (Fig. 3, cbs). Furthermore in the extracellular domain some of the putative N-glycosylation sites are highly conserved amongst the desmocollins (Fig. 3, marked with Ψ). In the cytoplasmic domain, homology is high near the transmembrane region and then decreases rapidly until the region prior to the splice site where conservation increases (Fig. 3, CYT). Beyond the alternative splice site, cytoplasmic tails of the a and b forms diverge dramatically; while all a forms are very similar to each other, they show little homology to the equally conserved set of b sequences (Fig. 3, CYTa and CYTb).

Comparison of the homologies of the protein sequence of bovine Dsc3 with other desmocollins (Table 1) indicates that it is most similar to the human HT-CP sequence, with a percentage identity of over 80%. The percentage identities of Dsc3 with Dsc2 (bovine, human and mouse) are around 60%, and with Dsc1 (bovine and human) only around 50%. Both the bovine Dsc3 and the human HT-CP contain a 43 bp exon which is different from the 46 bp exon found in the other isoforms. Also, both of them contain the putative adhesion site YAS in the N-terminal part of the protein. It therefore suggests that the human HT-CP is the human equivalent of bovine Dsc3, and that Dsc3 is the third isoform, similar to, but distinct from, previously described desmocollins.

Monoclonal antibody 07-4G is specific to Dsc3

We have previously described a monoclonal antibody, 07-4D, with specificity for bovine Dsc1 (Legan et al., 1994). Another antibody, 07-4G, similarly obtained by immunising mice with bovine desmosomal cores, appeared to show a staining pattern consistent with specificity for Dsc3. Staining sections of bovine epidermis with 07-4G showed that the antibody reacts strongly with the basal cell layer (Fig. 4). Reactivity decreased in the first suprabasal layer and, thereafter, showed a gradual further decrease until eventually disappearing in the spinous layer (Fig. 4). This staining pattern is consistent with the expression of DSC3 mRNA found by Legan et al. (1994), that is, strong expression in the basal layer diminishing fairly rapidly in the suprabasal layers.

The expression of DSC3 was shown by in situ hybridisation, northern blotting and RT-PCR to be strongly and specifically associated with the basal regions of stratified epithelia (Legan et al., 1994). The pattern of tissue reactivity of 07-4G was therefore studied. It was found that the antibody reacts with the basal cell layers of epithelia of oesophagus (Fig. 5A), rumen (Fig. 5B) and tongue (Fig. 6A). In each case, staining decreased in intensity and then disappeared in the intermediate layers of the epithelium. As indicated by staining with a monoclonal antibody, 11-5F, to desmoplakins, desmosomes are present to a level beyond the upper limit of 07-4G staining in each of these epithelia (Figs 5C,D and 6B), suggesting that the more apical

Table 1. Percentage amino acid identities of the 'a' and 'b' forms of mature desmocollins*

		BDsc1a	HDsc1a	BDsc2a	HDsc2a	MDsc2a	HT-CPa	BDsc3a	
		–	81.2) (86.9	52.8 (52.3)	52.6 (53.4)	53.7 (54.0)	53.9 (54.5)	55.6 (52.8)	BDsc1a
BDsc1b	–		–	52.3 (51.4)	54.7 (57.5)	53.6 (52.0)	51.4 (50.3)	51.9 (50.9)	HDsc1a
HDsc1b	80.5 (86.1)	–		–	76.4 (76.8)	73.3 (68.3	64.3 (68.9)	65.0 (68.1)	BDsc2a
BDsc2b	52.5 (48.4)	51.4 (44.4)	–		–	79.0 (75.7)	67.9 (81.9)	67.3 (77.9)	HDsc2a
HDsc2b	51.2 (51.6)	53.3 (50.8)	76.0 (75.6)	–		–	62.3 (68.9)	65.2 (67.5)	MDsc2a
MDsc2b	52.6 (48.4)	52.6 (45.2)	72.9 (64.4)	79.2 (64.4)	–		–	83.0 (83.4)	HT-CPa
HT-CPb	53.3 (53.7)	50.9 (48.8)	63.9 (69.4)	67.3 (83.5)	62.5 (70.2)	–		–	BDsc3a
BDsc3b	54.9 (52.5) BDsc1b	50.8 (47.6) HDsc1b	64.7 (66.1) BDsc2b	66.5 (77.2) HDsc2b	65.5 (68.5) MDsc2b	83.0 (85.1) HT-CPb	– BDsc3b		

*The figures are the percentage identity between the two sequences compared. Only the mature protein sequences of the 'a' and 'b' forms of the different isoforms of desmocollins are compared. The figures in brackets represent comparison between cytoplasmic domains of the two protein sequences.

HDsc2a and HDsc2b are human DGII/III sequences from Parker et al. (1991). Other sequence data: bovine Dsc1a/b (Collins et al., 1991), Dsc2a/b (Koch et al., 1992); mouse Dsc2a/b (Lorimer et al., 1994; King et al., 1993); human Dsc1a/b (Theis et al., 1993), Dsc2a/b (Parker et al., 1991) and HT-CPa/b (Kawamura et al., 1994).

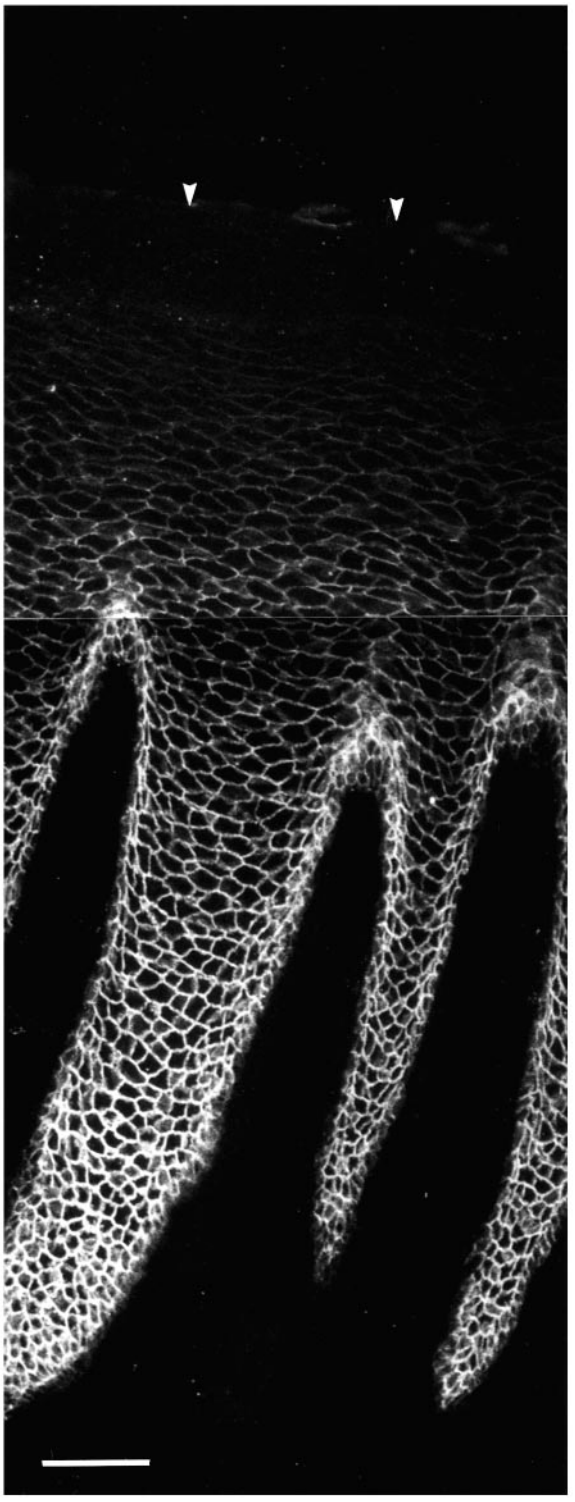

Fig. 4. Immunofluorescent staining of bovine nasal epidermis with 07-4G. The white arrowheads indicate the apical surfaces of the epidermis. Bar, 100 μm.

desmosomes do not contain Dsc3. All other bovine epithelial tissues tested - trachea, lung, liver, kidney, bladder - as well as cardiac muscle, were negative for 07-4G staining.

The isoform specificity of 07-4G was confirmed by western blotting. Firstly, the reactivity of 07-4G on bovine desmosomal core polypeptides was compared with that of the pan-desmocollin monoclonal antibody, 52-3D, and the Dsc1-specific monoclonal antibody, 07-4D (Fig. 7A). All these antibodies reacted with polypeptides of approximately the same electrophoretic mobility. Next, the reactivity of these three antibodies against fusion proteins of Dsc1, 2 and 3 expressed in *E. coli* was tested (Fig. 7B). This confirmed the pan-specificity of 52-3D, the Dsc1 specificity of 07-4D and indicated that 07-4G is Dsc3 specific.

To determine which part of the Dsc3 protein 07-4G recognises, fusion proteins expressing the extracellular domain and the cytoplasmic domains of the a and b forms were immunoblotted (Fig. 8). The results showed that 07-4G recognises an epitope in the extracellular domain of Dsc3.

DISCUSSION

We have obtained the complete cDNA sequence encoding the mature bovine Dsc3, both the a and b forms. We have also confirmed that expression of this isoform is restricted to the basal and lower suprabasal layers of stratified epithelia.

Our strategy for obtaining the sequence has been to extend it in steps by RT-PCR using a combination of sequence specific and redundant primers. This strategy is often employed in amplifying cDNA fragments which correspond to the protein of interest, and these are usually then used as probes to screen cDNA libraries to obtain clones covering the whole cDNA sequence. A problem of using PCR to elucidate nucleotide sequence is the possibility of copying error by Taq DNA polymerase and hence inaccuracy in the nucleotide sequence (Tindall and Kunjek, 1988). We have minimised this problem by sequencing *at least* four independent subclones, obtained from three separate PCR reactions, for each PCR reaction product. Although there were mismatches of certain bases between particular subclones, we had at least three identical copies for each base in the cDNA sequence. The reactivity of two monoclonal antibodies to native desmocollins with fusion proteins obtained from the cloned cDNA also supports the validity of our sequence.

We have previously tentatively suggested that bovine Dsc3 might be the homologue of the human desmocollin sequence known as DGII/III (Parker et al., 1991). This conclusion was based on percentage identity between the derived amino acid sequences of DGII/III and partial cytoplasmic domain of bovine DSC3, as well as similarity between in situ hybridisation patterns in epidermis (Arnermann et al., 1993; Legan et al., 1994). Comparison of the amino acid sequences of the cytoplasmic domains alone (Table 1) does indeed suggest homology between bovine Dsc3 and human DGII/III (called HDsc2 in Table 1). However, comparison of the complete sequences shows that DGII/III has considerably greater sequence identity to BDsc2 than BDsc3. We conclude that DGII/III is more likely to be the homologue of BDsc2 than BDsc3. Furthermore, a recently published human desmocollin sequence (HT-CP), obtained by Kawamura et al. (1994), was tentatively called human desmocollin type 4. However, our data demonstrated that it shows the greatest homology to bovine DSC3. Moreover, the homology between these two desmocollins is greater than that between any other pair of

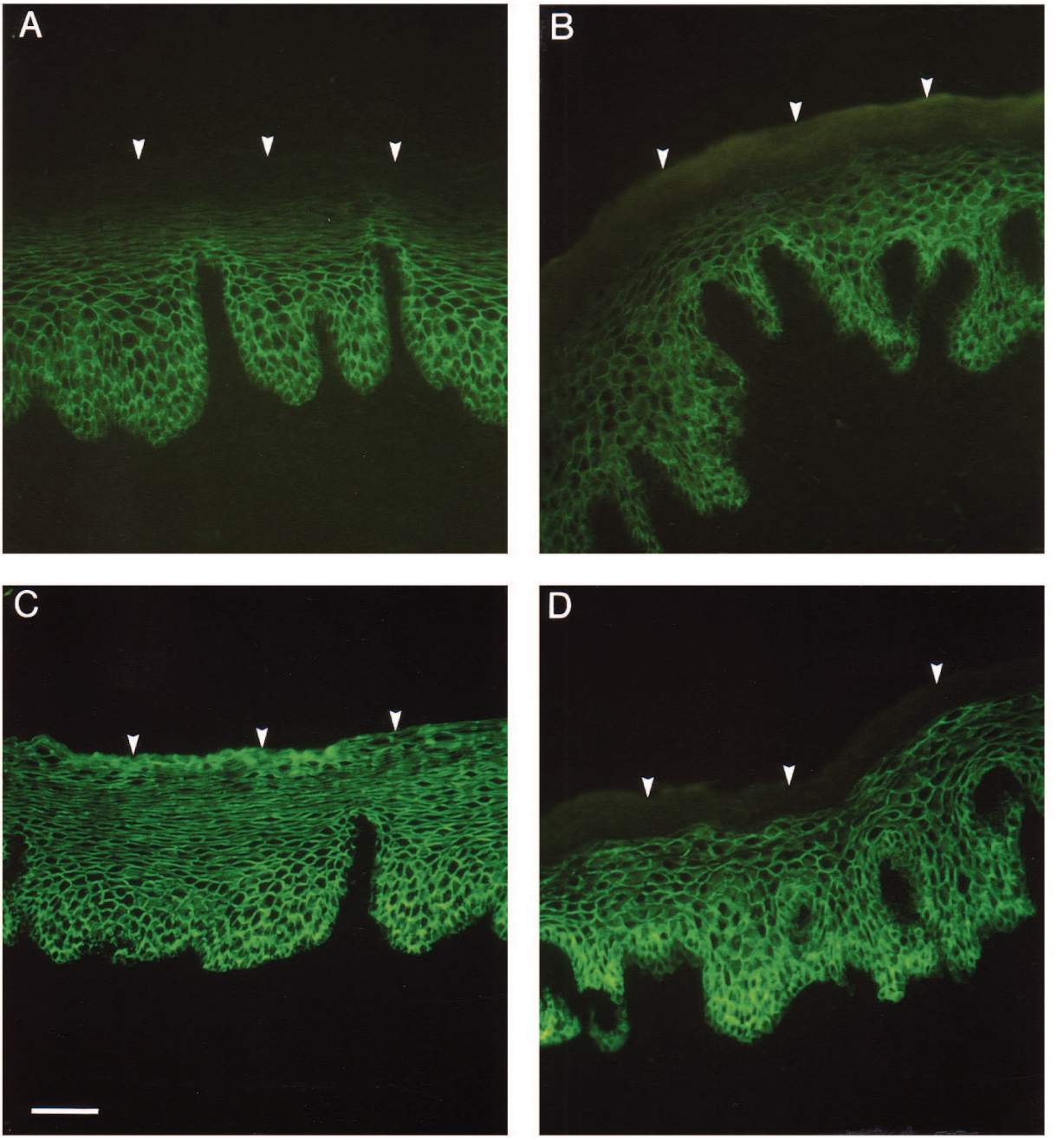

Fig. 5. Immunofluorescent staining of bovine oesophagus (A and C) and rumen (B and D) with 07-4G (A and B) and 11-5F (C and D). The white arrowheads indicate the apical surfaces of the epithelia. Bar, 100 μm.

desmocollins. Therefore we conclude that HT-CP is the homologue of BDsc3. Since bovine desmocollins are regarded as the standard for desmocollin nomenclature (Buxton et al., 1993; Legan et al., 1994) we propose that DGII/III should now be referred to as HDSC2 (for the gene) and HDsc2 (for the protein), and the HT-CP sequence as HDSC3 (for the gene) and HDsc3 (for the protein), respectively. This nomenclature for HT-CP has been mutually agreed between our groups and has been adopted in a paper recently submitted for publication (T. Hashimoto, T. Ebihara, M. Dmochowski, D. R. Garrod, K. Kawamura, T. Suzuki, S. Tsurufuji and T. Nishikawa). Thus three desmocollin isoforms have been found both in the bovine and human tissues.

Lorimer et al. (1994) have recently described the sequence of the first mouse desmocollin, MDsc2, bringing the total of known desmocollin sequences to seven. Alignment of the deduced sequences of the mature proteins (Fig. 3) reveals those regions showing greatest conservation and, therefore, presumably of greatest functional significance. These are discussed in detail by Lorimer et al. (1994). Here we shall make specific points of comparison between BDsc3 and other sequences. The most striking feature to emerge from sequence comparisons is

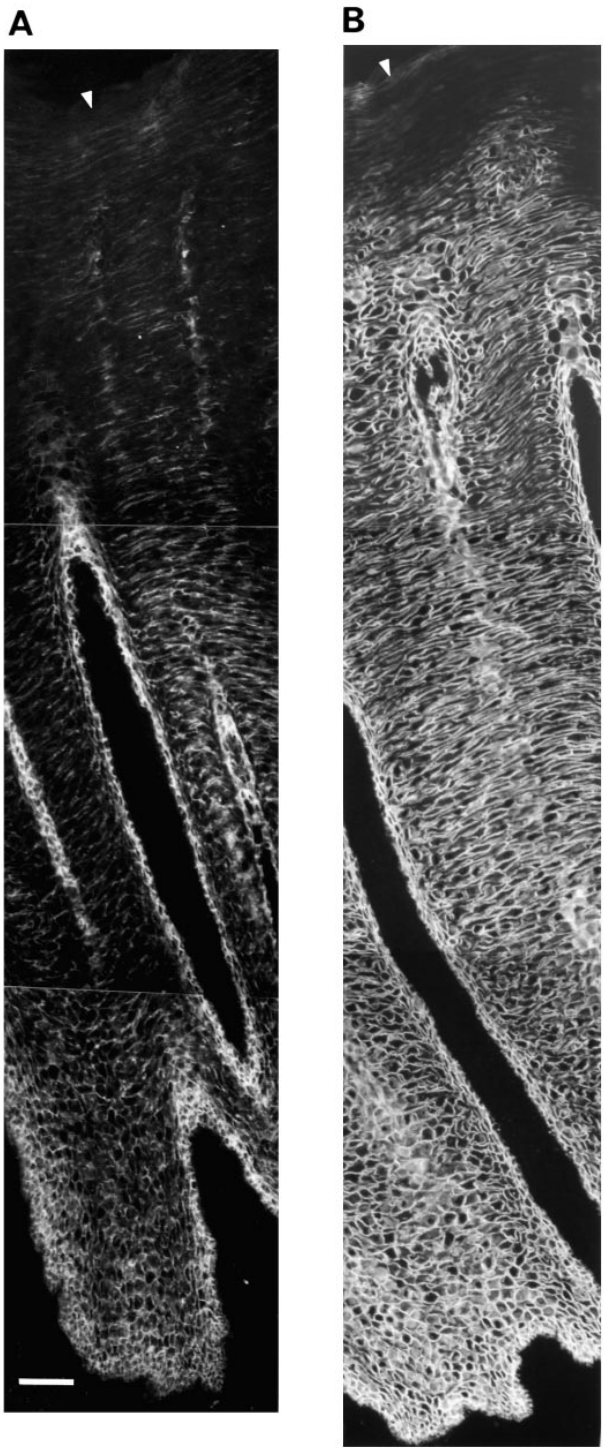

Fig. 6. Immunoflurorescent staining of bovine tongue epithelium with 07-4G (A) and 11-5F (B). The white arrow indicates the apical surface of the epithelium. Bar, 100 μm.

the very high degree of conservation of the N-terminal region of the extracellular domain. The degree of conservation gradually diminishes as the transmembrane region is approached. This conservation of primary structure is presumably associated with the function of desmocollins in cell-cell adhesion (Cowin et al., 1984). It is interesting that a short tripeptide sequence (HAV) near the N terminus is conserved in non-desmosomal cadherins and has been associated with a function in cell-cell adhesion (Blaschuk et al., 1990; Nose et al., 1990). The equivalent sequence is not conserved in desmocollins (Fig. 3). In both type 1 desmocollins (BDsc1 and HDsc1) it is represented by YAT and in all three type 2 desmocollins (BDsc2, HDsc2 and MDsc2) by FAT. In type 3 desmocollin (BDsc3 and HDsc3) the sequence is YAS. This presumably has some subtle significance in relation to the adhesive properties of the different desmocollin molecules.

Like other desmocollins, DSC3 shows alternative splicing in the cytoplasmic domain, thus encoding an ‘a’ form with a larger cytoplasmic domain and a ‘b’ form which is shorter (Collins et al., 1991; Parker et al., 1991). However, unlike other desmocollins which have an alternatively spliced exon of 46 bp and encode C-termini of 11 conserved amino acids, Dsc3 has an exon of 43 bp which encodes an 8 amino acid C terminus. The work of Troyanovsky et al. (1993) suggests that the cytoplasmic domain of the ‘a’ form possesses the essential binding properties for desmosomal plaque formation, but the ‘b’ form does not support plaque assembly. Thus the precise role of the ‘b’ form remains obscure, making it difficult to speculate about the reason for the even shorter cytoplasmic domain of Dsc3b. Since Dsc3 is most strongly expressed in basal regions, this domain may have some significance in relation to the functions of basal cells, such as proliferation or stratification.

Immunofluorescent staining with the isoform-specific monoclonal antibody, 07-4G, showed that Dsc3 is expressed only in stratified epithelia. This is consistent with the tissue distribution of Dsc3 mRNA as determined by northern blot and RT-PCR analysis, except that the latter indicated mRNA expression in tracheal epithelium (Legan et al., 1994). No protein was detected by antibody staining in tracheal epithelium, suggesting that the level of protein expression is very low or absent. This exclusive expression of Dsc3 in the more basal regions of stratified epithelia again suggests a specific function in relation to cell proliferation/stratification. Surprisingly, Dsc3 expression was also not detected in transitional epithelium of bladder (present results and Legan et al., 1994). This suggests some fundamental difference in the organisation of transitional and stratified epithelia.

Both 07-4G staining and in situ hybridisation (Legan et al., 1994) indicate that Dsc3 is expressed most strongly, but not exclusively, in basal cells, and the intensity of expression appears to fade gradually in the suprabasal layers. This contrasts with the expression of basal keratins, K5 and K14, and of integrins, where expression ceases more or less abruptly above the basal layer (Kopan and Fuchs, 1989). Moreover, comparison of the expression patterns of Dsc1 and Dsc3 in epidermis (compare Fig. 8 of Legan et al., 1994 with Fig. 4 here) shows a substantial region of overlap between the regions where the two isoforms are expressed. We do not yet have a monoclonal antibody specific for Dsc2, but in situ hybridisation suggests that its expression overlaps both Dsc1 and Dsc3 in epidermis, and Dsc3 in other stratified epithelia (Legan et al., 1994). These results suggest that individual cells possess quantitatively different ratios of the desmocollin isoforms dependent upon what level of stratification they occupy. Thus the adhesive properties of the cells may form a vertical gradient which may be instrumental in determining cell position, and/or

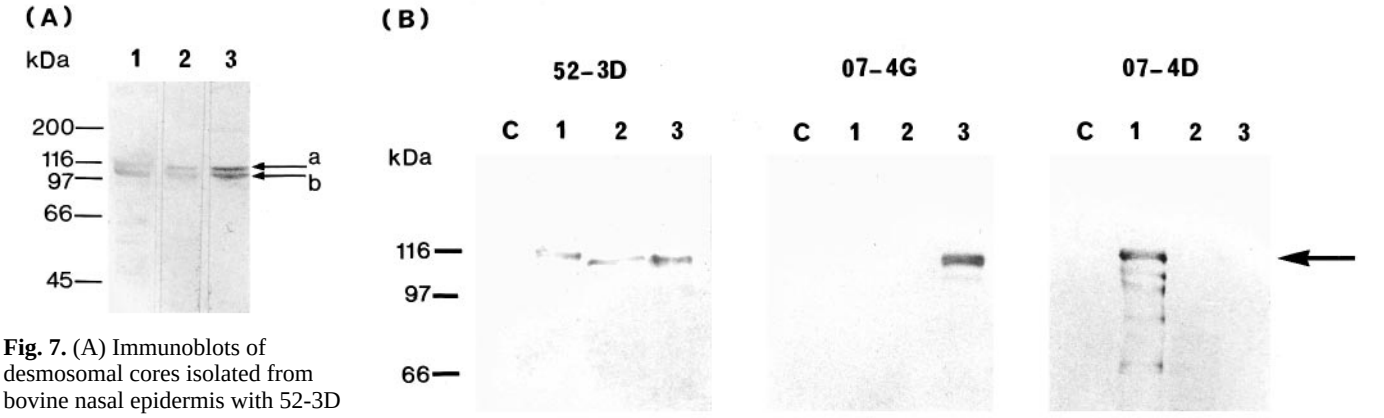

Fig. 7. (A) Immunoblots of desmosomal cores isolated from bovine nasal epidermis with 52-3D (lane 1), 07-4G (lane 2) and 07-4D (lane 3). The positions of the a and b forms of the desmocollins are arrowed. (B) Immunoblots of bacterial cell lysates of IPTG-induced pGEX2T, the control (lanes C), pGEX3X/Dsc1b (lanes 1), pGEX4T/Dsc2b (lanes 2) and pGEX2T/Dsc3b (lanes 3) transformants with monoclonal antibodies 52-3D, 07-4G and 07-4D. The positions of the Dsc1, Dsc2 and Dsc3 fusion proteins are arrowed. Lower molecular mass bands represent breakdown products of the fusion proteins.

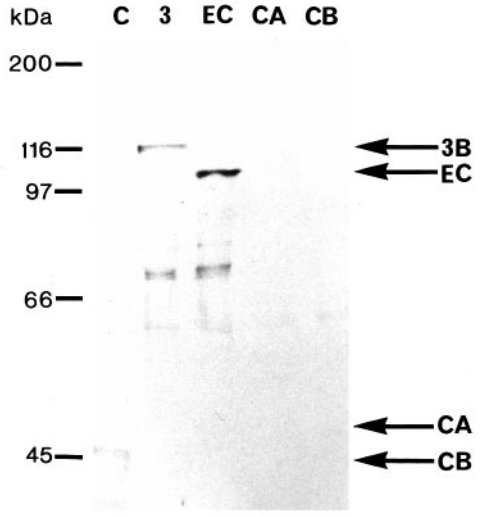

Fig. 8. Immunoblots of bacterial cell lysates of IPTG induced pGEX2T (lane C), pGEX2T/Dsc3b (lane 3), pGEX2T/Dsc3-EC (lane EC), pGEX2T/Dsc3a-CYT (lane CA) and pGEX2T/Dsc3b-CYT (lane CB) transformants with monoclonal antibody 07-4G. (pGEX2T/Dsc3b encodes the fusion protein containing complete Dsc3b (BDsc3b), pGEX2T/Dsc3-EC encodes the fusion protein containing the extracellular domain of Dsc3 (EC), pGEX2T/Dsc3a-CYT and pGEX2T/Dsc3b-CYT encode fusion proteins containing cytoplasmic domains of Dsc3a (CA) and Dsc3b (CB) respectively, pGEX2T is the control). The positions of the fusion proteins (BDsc3b, EC, CA and CB), determined from Coomassie Blue-stained gels (not shown), are arrowed.

signalling state of differentiation. The availability of antibodies specific for two desmocollin isoforms may allow us to study the protein expression of the isoforms in different tissues and their regions of overlap in more detail. Using immunoelectron microscopy we are now able to investigate whether cells contain individual desmosomes with distinct desmosomal isoforms or whether individual desmosomes contain variable mixtures of the different isoforms.

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Note added in proof

A confirmatory study by Nuber et al. (1995, *Eur. J. Cell. Biol.* 66, 69-74) supports the data previously published by us (Legan et al., 1994) on the distribution of desmocollin isoform expression in mammalian tissues, as well as the data of Dsc3 expression contained in the present paper. Interestingly, Nuber et al. also found Dsc3 expression in a squamous cell carcinoma of lung and a transitional cell carcinoma of kidney.