Topography of Mammalian Connexins in Human Skin

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We have explored the expression of gap junction proteins in normal human skin by immunostaining cryostat sections (indirect immunofluorescence) or lyophilized epidermis (Western blotting) with antibodies against four mammalian connexins Cx26, Cx32, Cx40, Cx43; and by hybridizing total epidermal RNA (Northern blotting) with cRNA probes for Cx26, Cx32, and Cx43. We found that epidermal keratinocytes express Cx43 but not Cx26, Cx32, or Cx40. This expression was minimal in the basal layer, much higher in the spinous layer, reduced in the granular layer, and absent in the stratum corneum. Immunostaining for Cx43 was also observed in sebaceous glands, hairs, and eccrine sweat ducts. The two latter epidermal adnexae were also markedly labeled by antibodies against Cx26, a gap junction protein that was undetectable by immunofluorescence in interfollicular keratinocytes. Immunoblots of polyacrylamide gel electrophoresis – separated epidermal proteins and hybridization of epidermal RNA confirmed the presence of Cx43 in epidermis.

These observations indicate that 1) Cx43 and Cx26 are components of human keratinocyte gap junctions; 2) these two proteins are differentially expressed in the interfollicular epidermis and the skin adnexae; 3) in interfollicular epidermis, Cx43 is a predominant gap junction protein, mostly expressed by the differentiating spinous cells; 4) Cx43 distribution is in accordance with the extensive dye coupling previously observed in this epidermal compartment. *Key words: gap junctions/keratinocytes/coupling/epidermis/epidermal adnexae. J Invest Dermatol 103:240 – 247, 1994*

A form of intercellular communication operates via highly permeable membrane channels that permit the exchange of small metabolites and ions between neighboring cells [1,2]. These channels are gap junctions, which are formed by two hemichannels, or connexons, which join in mirror symmetry. Each connexon is itself formed by six peptides that cross the plasma membrane four times, have their N- and C-termini in the cytoplasm, and limit a central hydrophilic space [3]. The proteins that form connexons have been recently identified as members of a family of non-glycosylated integral membrane proteins, which have been called connexins (Cx) [4 – 6]. It is now clear that gap junctions in various tissues are formed by different connexins [4,5]. This differential composition may impart specific characteristics to gap junction channels that, in turn, could be essential for different physiologic functions.

Human epidermal keratinocytes are connected by gap junctions [7 – 11], which presumably ensure the cell-to-cell diffusion of electrotonic currents [12] and low – molecular-weight tracers across the intact epidermis [13] and in culture [14,15]. Recently, two reports have documented that these cells are stained by antibodies to Cx43 [16,17]. However, the cellular structures immunolabeled were not investigated, and a somewhat different evaluation of the levels and distribution of Cx43 across the epidermis was provided. Furthermore, these two studies failed to detect other connexins in interfollicular epidermis, a finding contrasting with the previously reported colocalization of Cx43 and Cx26 in rat epidermis and skin adnexae [18] or in primary cultures of mouse keratinocytes [19]. Whether this major difference reflects a species specificity or rather a change in the connexin pattern as a function of keratinocyte localization, differentiation, and/or environment remains to be elucidated.

To address these questions, we have investigated normal human skin using antibodies and cRNA probes to Cx26, Cx32, Cx40, and Cx43, four of the best characterized proteins of the twelve that are now known to comprise mammalian gap junctions [4 – 6].

MATERIALS AND METHODS

Tissue Keratome samples of normal human skin [13] were obtained from specimens of either breast or abdominal reduction surgery as well as from the neck of control volunteers after informed consent of the patients and in agreement with the guidelines of our institutional committee for clinical investigation.

Fragments of abdominal and footpad skin were sampled from normal swiss type adult mice immediately after sacrifice.

Immunohistochemistry For fluorescence microscopy, skin fragments were rapidly frozen by immersion in 2-methylbutane that was cooled with liquid nitrogen and stored at −80°C until histologic processing. Frozen sections of about 5-μm thickness were cut with a cryomicrotome (1720 digital MGW Leitz, Germany), collected on gelatin-coated slides, and fixed 3 min in −80°C acetone. All slides were then rinsed in cold (4°C) phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and processed for indirect immunofluorescence. For localization of gap junction proteins, sections were first incubated for 2 h at room temperature with one of the following antibodies: 1) affinity-purified rabbit polyclonal against liver Cx32, diluted 1 : 100 [20]; 2) monoclonal R5.21 C against liver Cx32, undiluted culture supernatant [21]; 3) affinity-purified rabbit polyclonal against residues 108 – 117 of liver Cx26, diluted 1 : 30; 4) affinity-purified rabbit polyclonal against residues 101 – 119 of liver Cx26, diluted 1 : 100 (courtesy of Dr. Shibata); 5) affinity-purified rabbit polyclonal against mouse liver Cx26 [22]; 6) polyclonal rabbit antiserum against residues 252 – 271 of heart Cx43, diluted 1 : 100 [23]; 7) affinity-purified rabbit antibodies against residues 314 – 322 of heart Cx43, diluted 1 : 100 [24]; 8) polyclonal rabbit serum against residues 313 – 330 of Cx40 [25]; or 9) polyclonal rabbit serum against residues 346 – 358 of Cx40 [26]. Sections were then rinsed in phosphate-buffered saline (PBS) and incubated 1 h at room temperature

Manuscript received May 24, 1993; accepted for publication February 14, 1994.

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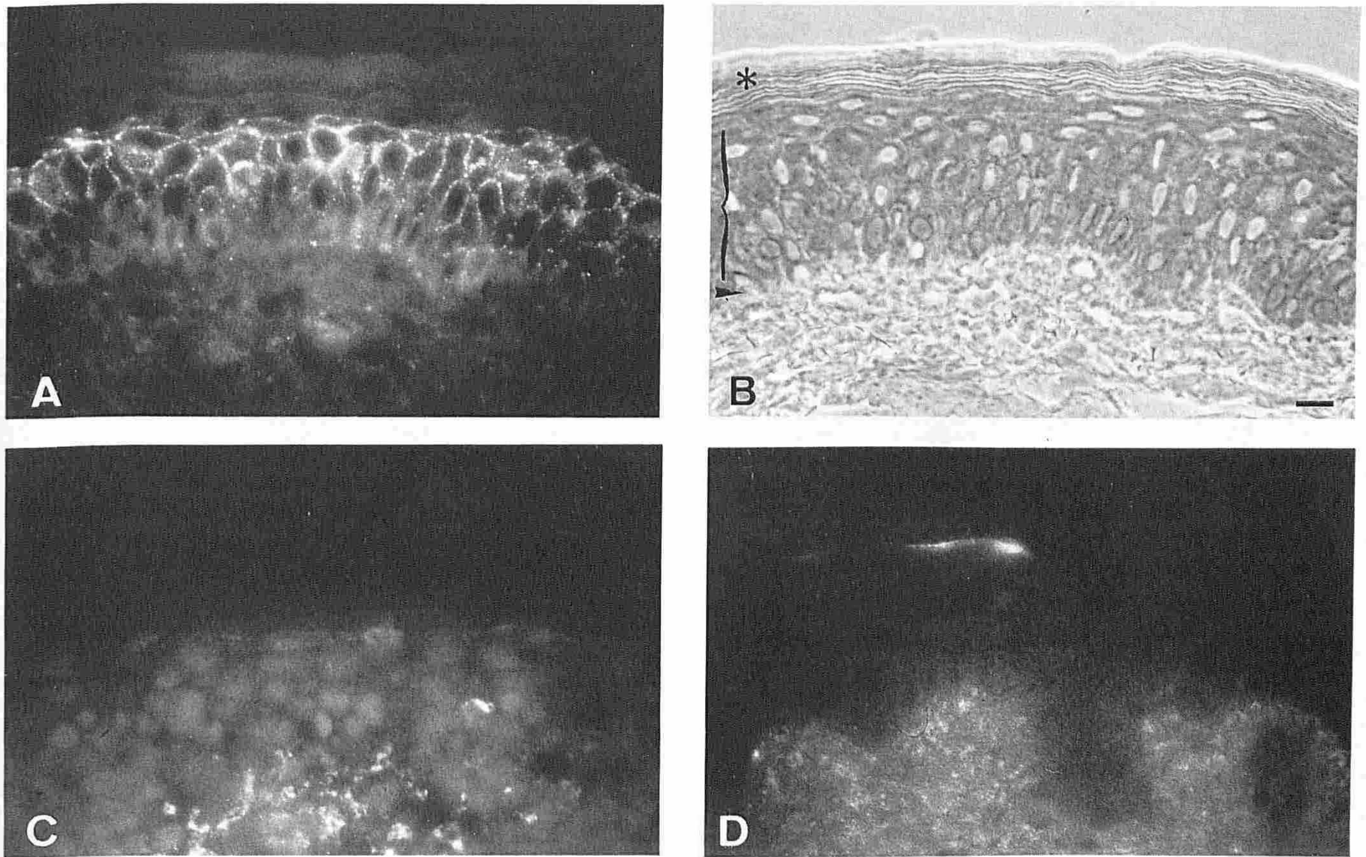

Figure 1. Immunolabeling of human skin for connexins. Incubation of a skin cryosection with polyclonal sera against Cx43 *(A)*, Cx26 *(C)*, and Cx32 *(D)*. *A)* With an antiserum against Cx43, the labeling was scarce in the basal layer, much more abundant in the spinous and granular layers, and absent in the stratum corneum. *B)* The phase-contrast view of the section shown in *A* illustrates the topography of the basal *(arrowhead)*, the spinous *(bracket)*, and the cornified layers *(asterisk)*. *C)* No specific immunostaining of human skin was seen with an antiserum against Cx26. *D)* Similar negative observations were made with antibodies to Cx32. (*Bar*, 12 μm.)

with either fluorescein-conjugated anti-rat or anti-rabbit antibodies, whichever applicable, diluted 1:200. After further rinsing, sections were covered with 0.02% paraphenylenediamine in PBSglycerol (1:2, v:v) and photographed on a Axiophot microscope (Zeiss, Oberkochen, West Germany) fitted with filters for fluorescein detection.

Quantitative evaluation of Cx43 immunostaining was performed on samples from four volunteers. To this end, 10 regions of interfollicular epidermis were photographed at a magnification of 157× in the sections obtained from each volunteer. Color slides of these sections were projected on a graphic tablet at the final magnification of ×1500. The number of immunofluorescent spots and of cells was scored separately in the basal, spinous, granular, and stratum corneum layers of the epidermis. From these data, the number of immunolabeled spots per cell was calculated in each epidermal compartment and expressed as mean ± SEM.

For electron microscopy, skin samples were fixed at room temperature in a 0.1% glutaraldehyde–4% paraformaldehyde mixture for 5 min and then exposed 55 min to 4% paraformaldehyde. The fragments were then embedded at low temperature in Lowicryl K4M resin and thin sectioned using a Reichert OM10 ultramicrotome. Sections were first incubated 15 min in PBS containing 0.1% bovine serum albumin and then exposed 2 h at room temperature to polyclonal rabbit antibodies, diluted 1:10 (number 7 in the above list). After being rinsed, sections were further exposed 1 h at room temperature to a swine anti-rabbit and gold-conjugated antibody (Sigma Chemical Co., St Louis, MO), diluted 1:100. Sections were then photographed on a CM10 electron microscope (Philips, Eindhoven, The Netherlands).

In all immunostaining experiments, controls included exposure of sections during the first incubation to either preimmune sera, or to sera that had been preabsorbed with the peptide used for immunization (whichever available), and to the fluorescein or gold-conjugated antibodies that were normally used during the second incubation step. As positive controls, mouse or rat liver, heart, and skin sections were used for the different Cx tested.

Western Blotting Immediately after keratome sampling (300 μ thick) on breast or abdominal surgery specimens the skin was cut into fragments of approximatively 5 × 20 mm and incubated 30 min at 4°C, under gentle agitation, in a PBS prepared without adding Ca^{++} and supplemented with 0.1 mg/ml thermolysin (Sigma, Chemical Co., St Louis, MO). Under control of a binocular microscope, the epidermis was then splitt from the dermis using fine forceps and collected in cold (4°C) 0.02 M Tris-HCl buffer (pH 7.8) supplemented with 20 mM ethylenediaminetetraacetic acid (EDTA), 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 mM benzamidine, 200 Kin/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 1 mM DFP. Epidermal fragments collected within the 2 h that followed skin surgery were frozen, pulverized under liquid nitrogen, freeze-dried, and stored at −80°C. Aliquots (1 mg) of lyophilized epidermis were solubilized by a 30-second sonication in 0.06 M Tris-HCl buffer (pH 6.8), supplemented with 20% sodium dodecylsulfate (SDS), 20 mM EDTA, and 5% 2-mercaptoethanol and kept at least 1 h in this buffer at room temperature. Protein content was measured by the Schaffner and Weissman method [27]. Equivalent amounts of protein (20 μg/lane) were fractionated by electrophoresis in a 12.5% SDS-polyacrylamide gel and then transferred on 0.22 μm Immobilon membranes (Millipore, MA) for 18 h at 20 V, using a 25-mM Tris-HCl buffer containing 192 mM glycine, 20% methanol, and 0.02% SDS. After being checked for efficient transfer by Ponceau-S staining, the membranes were saturated for 1 h in 40 mM Tris-HCl (pH 7.5), supplemented with 0.1% SDS and 5% dry milk (BLOTTO solution) [28], and incubated for 2 h at room temperature with one of the affinity-purified antibodies listed above diluted in Blotto solution (1:100 for anti-Cx43 antibody number 7, 1:50 for anti-Cx26 antiserum number 3, 1:20 for anti-Cx32 antiserum number 1). Following repeated rinsing in 40 mM Tris HCl containing 0.1% Tween 20 and 4% dry milk, the immunoblots were incubated 1 h at room temperature with a biotinylated donkey anti-rabbit serum (Amersham, Amersham, England) diluted 1/500, rinsed, and incubated again 1 h at room temperature with a streptavidin alkaline-phosphatase conjugate (Amersham), diluted

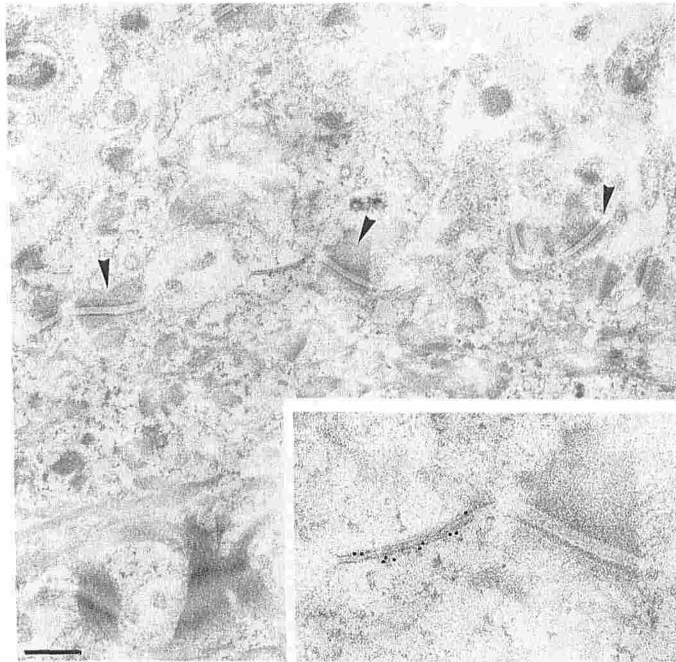

Figure 2. Immunolabeling of keratinocyte gap junctions. Immunoelectromicroscopy of human epidermis embedded in Lowicryl K4M resin with a polyclonal antibody to Cx43. The cytoplasmic side of membrane appositions typical of gap junctions were labeled by the antiserum against Cx43, as demonstrated by the presence of 10-nm gold particles conjugated to a second antibody. Note the absence of gold particles on other organelles, including bundles of keratin filaments and desmosomes *(arrowheads)*. The abundance of these two organelles identify the two interacting cells shown here, as keratinocytes of the spinous layers. (*Bar*, 240 nm, 80 nm in *inset*.)

1/8000. Detection of alkaline-phosphatase activity was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [29].

In all blots, controls included omission of the affinity-purified antibodies during the first incubation and incubations with antibodies that had been previously incubated with the synthetic peptide against which they were raised.

Table I. Distribution of Connexins in Control Human Skin[a]

	Cx 43	Cx 26	Cx 32	Cx 40
Interfollicular epidermis				
Stratum corneum	–	–	–	–
Stratum granulosum	++	–	–	–
Stratum spinosum	++++	–	–	–
Stratum basale	+	–	–	–
Hair follicles				
Outer root sheat	+++	++	–	–
Inner root sheat				
Henley	++	++	–	–
Huxley	++	++	–	–
Cuticle	–	–	–	–
Hair shaft				
Cuticle	–	–	–	–
Cortex	++	++	–	–
Medulla	++	++	–	–
Matrix	+	+	–	–
Dermal papillae	++	–	–	–
Sebaceous glands	++++	–	–	–
Eccrine sweat glands (ducts)	+	++++	–	–
Dermis	+	–	–	–

[a] + and – indicate the presence and absence of connexins immunoreactivity, respectively.

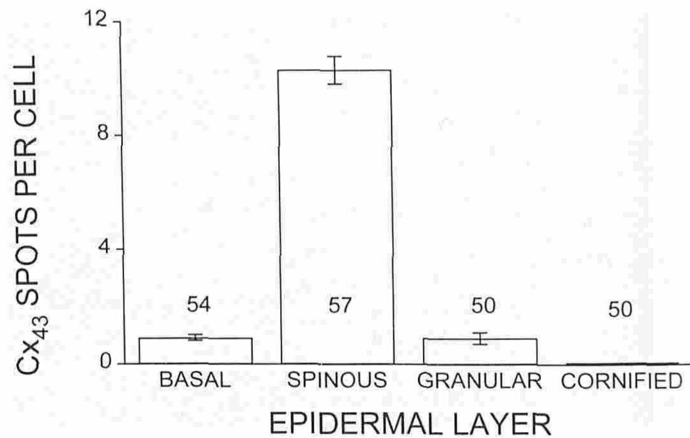

Figure 3. Cx43 distribution in the epidermis. Quantitative evaluation of Cx43 immunostaining was performed by separately scoring the number of immunofluorescent spots per cell in the basal, spinous, granular, and stratum corneum layers. Values are mean ± SEM of the number of epidermal regions indicated.

A similar protocol was followed to prepare and blot human heart and liver, as well as rat heart and brain, which were used as internal controls in every immunoblot.

Northern Blotting Total cellular RNA was prepared by homogeneizing fragments of keratomed skin from breast or abdominal surgery or enzymatically split epidermis and dermis in 2.5 ml of 0.1 M Tris-HCl, pH 7.4, containing 1 M β-mercaptoethanol and 4 M guanidinium thiocyanate. After addition of solid CsCl (0.4 g/ml), the homogenate was layered onto 2 ml of a

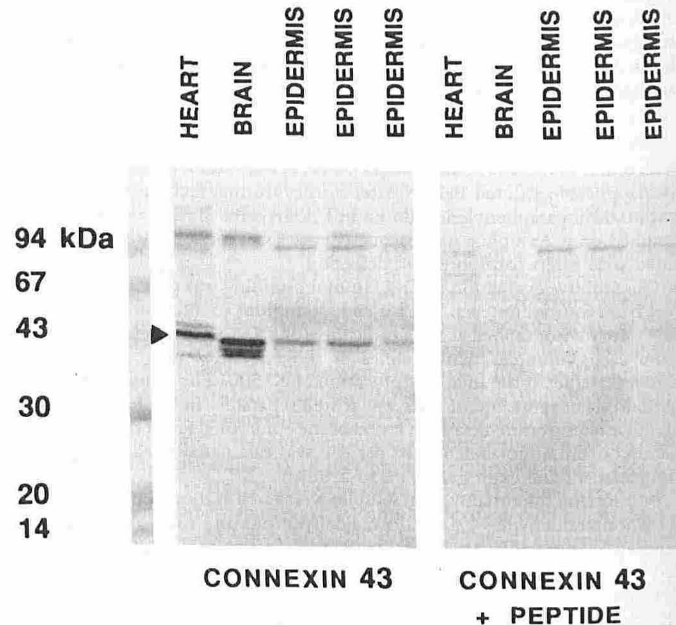

Figure 4. Immunoblot of Cx43 in human epidermis. Immunoblot analysis of Cx43 in human heart, rat brain, and human epidermis. Proteins were separated by a 12.5% SDS-polyacrylamide gel electrophoresis, electroblotted to Immobilon membranes and probed with polyclonal antibodies to residues 314–322 of Cx43. *Left:* immunoblot revealed a band of 43 kDa in human heart and two specific bands with slightly faster electrophoretic mobility (41–42 kDa) in rat brain. A band of 42 kDa, which had a similar electrophoretic mobility of one of the brain proteins, was detected in epidermis. *Right:* the specific bands at 41–43 kDa were no longer detected after preabsorbtion of the antibodies with the immunizing peptide. All lanes were loaded with about 20 μg proteins. The position of the molecular weight markers is shown on the *left* lane.

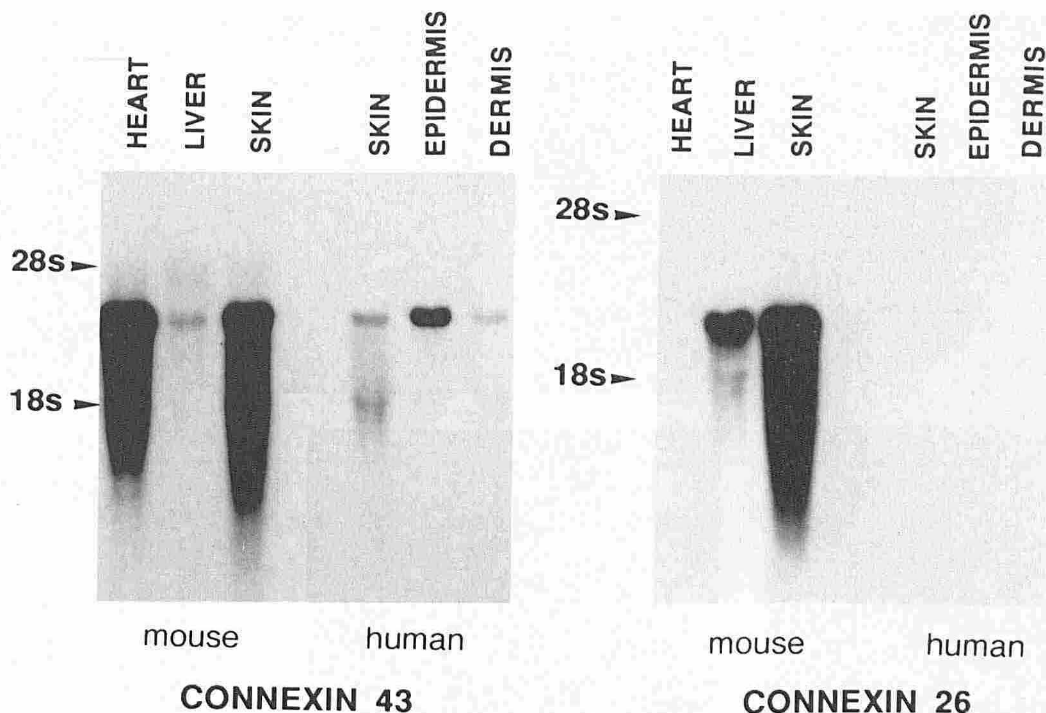

Figure 5. Hybridization blots of connexin mRNA from mouse and human skin. Northern blot analysis of total RNA isolated from mouse tissues (heart, liver, skin) and human skin with a ^{32}P-labeled cRNA probe for Cx43. *Left:* an abundant transcript of about 3.0 kb was detected in mouse heart and, to a much lesser extent, in mouse liver. The same probe also hybridized to a single mRNA in extracts of intact mouse and human skin. The 3.0-Kb transcript was predominantly expressed in the epidermis and was barely detectable in the dermis. *Right:* an analogous screening with a cRNA probe for Cx26 revealed a single specific transcript in mouse liver and skin that was not detected in mouse heart. No such transcript was detected in intact human skin, epidermis and dermis. All lanes were loaded with 5 μg total RNA.

5.7 M CsCl-0.1 M EDTA (pH 7.4) cushion and centrifuged in a Beckman SW55 rotor at 35,000 rps for 20 h at 20°C. Pelleted RNA was resupended in 10 mM Tris-HCl, pH 8.1, supplemented with 5 mM EDTA and 0.1% SDS, extracted twice with phenol-chloroform, precipitated in ethanol, and resuspended in water.

A probe for Cx32 was prepared by subcloning a 504 bp EcoRI-SmaI fragment of a 1.5-Kbp rat liver gap junction cDNA [30] into plasmid pSP64 [31]. A probe for Cx43 was constructed by subcloning a 1.4-Kbp fragment (clone G2A) of a rat heart gap junction cDNA into the EcoRI site of Bluescript M13 [32]. A probe for Cx26 was constructed by subcloning a 600-bp Pstl-EcoRI fragment of a 1.1-Kbp rat liver gap junction cDNA [33] in plasmid pSP65. Plasmids pSP64 Cx32, pBSG2A, and BSP65 Cx26 were linearized using EcoRI and BamHI and Pstl, respectively, and used as templates for bacteriophage SP6 (in the case of pSP64 Cx32 and pSP65 Cx26) or T3 RNA polymerase (in the case of pBSG2A). Transcription was performed as previously described [34].

For Northern blots, total cellular RNA was denatured with glyoxal, electrophoresed in a 1.2% agarose gel (5 μg total cellular RNA/lane), and transferred overnight onto nylon membranes (Hybond, Amersham), as previously described [35]. Filters were baked under vacuum at 80°C for 2 h and stained with methylene blue to reveal 18S and 28S rRNA species. Filters were boiled 5 min in 20 mM Tris-HCl, pH 8.1, to remove residual glyoxal, prehybridized 6 h at 65°C and then hybridized 18 h, at the same temperature, with 2×10^6 cpm/ml of ^{32}P-labeled probes [34]. The filters were washed twice at 65°C with $3 \times$ SSC solution (SSC solution is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), then with $2 \times$ Denhardt's solution and, eventually, three times at 75°C with a $0.2 \times$ SSC solution supplemented with 0.1% SDS and 0.1% sodium pyrophosphate. Filters were exposed to Kodak XAR-5 films between intensifying screens at −80°C for 6 d.

RESULTS

Connexins in Interfollicular Epidermis Incubations of cryosections with two different antisera against Cx43 resulted in the immunolabeling of numerous spots on keratinocyte membranes (Fig 1*A*). At the electron microscopy level, this labeling was seen to correspond to the cytoplasmic sides of close membrane appositions typical of gap junctions and was virtually excluded from all other organelles including desmosomes and bundles of intermediate keratin filaments (Fig 2). Immunolabeling of Cx43 was absent on the portion of the keratinocyte membrane that contacted the basal lamina, minimal on the lateral plasma membranes of basal keratinocytes, very pronounced all around keratinocytes of the spinous layers, reduced in the granular layers, and absent in the stratum corneum (Fig 1*A,B* and Table I). This heterogeneous distribution was confirmed by a quantitative analysis, which revealed that the distribution of immunofluorescent spots given by an anti-Cx43 serum was small in basal keratinocytes, much higher in keratinocytes of the spinous layers, abruptly decreased in granular keratinocytes, and nil in keratinocytes of the uppermost cornified layers (Fig 3).

Western blotting of human heart samples with polyclonal antibody to residues 314-322 of Cx43 revealed a predominant band of 43 KDa. In rat brain, the same antibodies markedly labeled two specific bands that had a slightly faster electrophoretic mobility (apparent molecular weight of 41-42 kDa) than the protein detected in heart (Fig 4). These two organs, which are known to express Cx43, were used as internal controls. In three different samples of enzymatically splitted epidermis, a band with electrophoretic mobilities similar to those observed in brain extracts were also detected by the anti Cx43 serum (Fig 4). These bands were no longer detected when the antibodies were preabsorbed with the peptide that had been used for immunization (Fig 4). Under the sensitive conditions we used, moderate staining of other protein bands, including keratins, was also observed. However, this staining was considered non-specific, because it was not modified by preabsorption of the antiserum with peptides used for immunization (Fig 4).

In Northern blots of total skin RNA extracted from abdominal skin, an area with a low density of epidermal adnexae, a riboprobe specific for Cx43 hydridized with a single mRNA species that had the same size (about 3.0 kb) and mobility of the transcript detected in heart samples but was much less abundant (Fig 5). The levels of this Cx43 transcript were significantly lower in intact skin than in

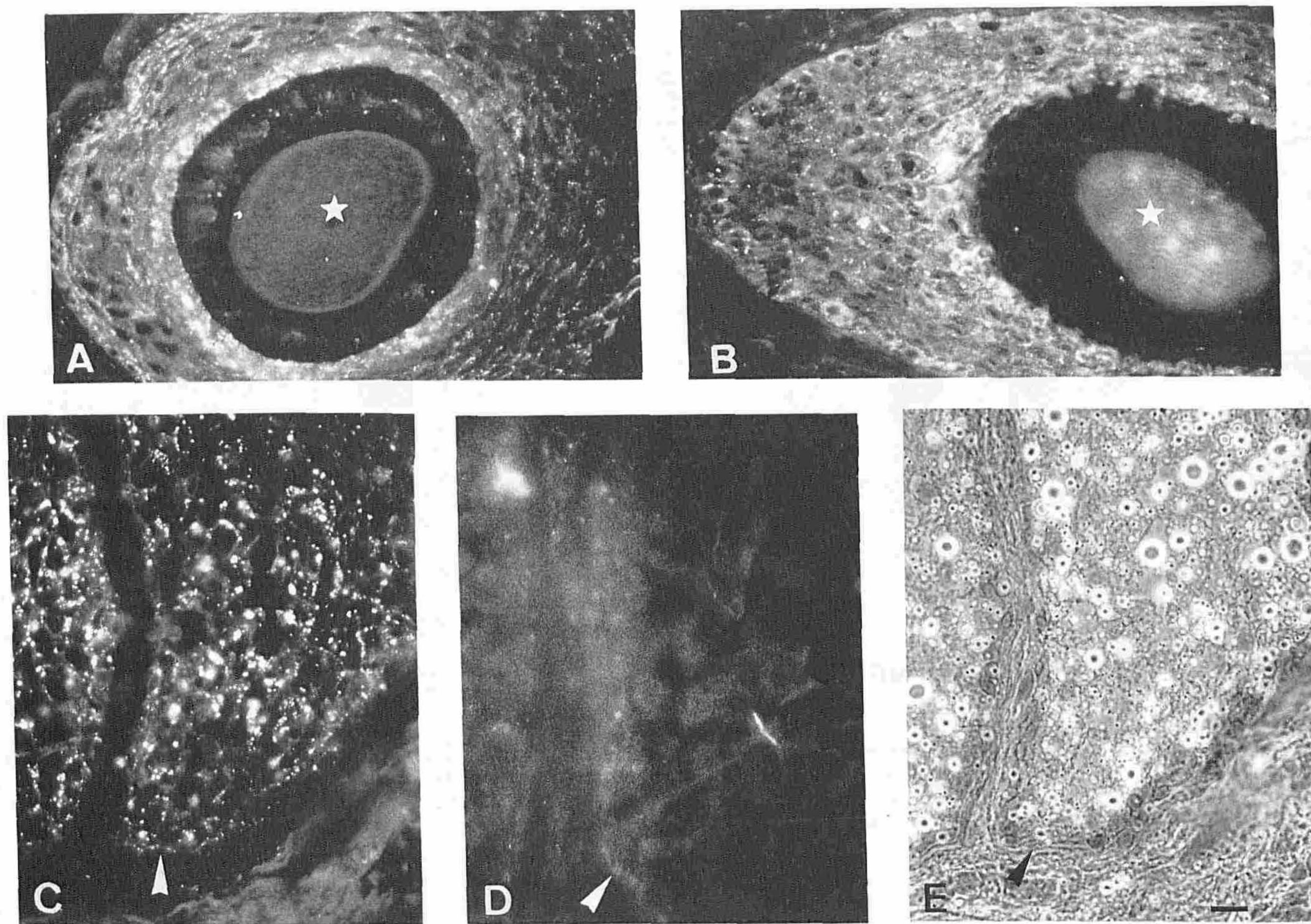

Figure 6. Immunolabeling of pilosebaceous apparatus for connexins. Incubation of a skin cryosection with polyclonal sera against Cx43 *(A,C)* and Cx26 *(B,D)*. *A,B)* The inner and outer root sheaths of hair follicles, but not the hair shafts *(stars)*, were markedly labeled by sera against Cx43 *(A)* and Cx26 *(B)*. *C,D)* In sebaceous glands, antisera to Cx43 *(C)*, but not to Cx26 *(D)*, resulted in an abundant immunolabeling of sebocytes. *E)* The phase-contrast of the section shown in *C* reveals that the Cx43 immunostaining predominated in the differentiated sebocytes. A less abundant, punctate staining was also observed in the basal layer *(arrowhead)*. *(Bar,* 20 μm in *A* and *B* and 12 μm in *C, D,* and *E.)*

epidermis (Fig 5). The same probe also hydridized to a single mRNA in extracts of intact mouse skin. Enzymatic splitting of the human epidermis revealed that the 3.0-Kb transcript was predominantly expressed in this tissue and was barely detectable in the dermis (Fig 5).

Similar experiments were performed to search for the expression of Cx26. With three specific antibodies, this gap junction protein was readily identified in mouse epidermis (not shown) but not in epidermis of normal human skin (Figs 1*C* and 5). A screening with a cRNA probe for Cx26 revealed a single specific transcript in mouse liver and skin that was not detected in mouse heart. No such transcript was detected in intact human skin epidermis and dermis, even after prolonged exposure of the autoradiographs (Fig 5).

Similarly, no Cx32 was detected in human interfollicular epidermis by either immunofluorescence (Fig 1*D*), Western or Northern blotting (not shown). Immunofluorescence labeling also did not show Cx40 within human epidermis (Table I).

Connexins in Epidermal Adnexae The presence of connexins in sebaceous glands, hair follicles, and eccrine sweat glands was screened by immunofluorescence. This screening did not detect Cx32 and Cx40, but revealed high levels of Cx43 and Cx26, which were variably distributed in different epidermal adnexae (Table I).

Within sebaceous glands, antibodies to Cx43 resulted in a modest, punctate staining of the peripheral, non-differentiated cell layer, and in a much more abundant and coarse labeling of the more centrally located, differentiating cell layers (Fig 6*C,E*, and Table I). Freeze-fracture and immunoelectron microscopy confirmed that the latter labeling resulted from the presence between fully differentiated sebocytes of numerous and large gap junctions, which often had an annular configuration (not shown). In contrast, the different antibodies against Cx26 failed to detect this gap junction protein in both basal and fully differentiated sebocytes (Fig 6*D* and Table I).

In the hair follicles (most of which were in the anagen stage of development), both outer and inner root sheaths were markedly stained for Cx26 and Cx43 (Fig 6*A,B* and Table I). This marked immunostaining contrasted with the absence of any specific labeling over the central, fully keratinized hair shaft (Fig 6*A,B* and Table I). Cx26 and Cx43 were also coexpressed in the epidermal portion of the hair follicle matrix, whereas only Cx43 was detected in the nearby dermal papillae (Table I).

The excretory ducts of eccrine sweat glands were stained by antibody to Cx43 (Fig 7*F* and Table I) and Cx26 (Fig 7*A–E* and Table I) throughout their length, i.e., from the lower region deep in the reticular dermis (Fig 7*A,B*) to the region crossing the interfollicular epidermis (Fig 7*C,D*). At all levels, the labeling for Cx26 was strong over the two concentric layers of cells that comprised the wall of the ducts (Fig 7*E*). In contrast, the labeling for Cx43 was less marked and predominated over the central layer of cells that bordered the lumen of the ducts (Fig 7*F*). No specific connexin immunostaining was detected in the secretory coils of eccrine sweat glands, which displayed an intense autofluorescence (Fig 7*A,B*).

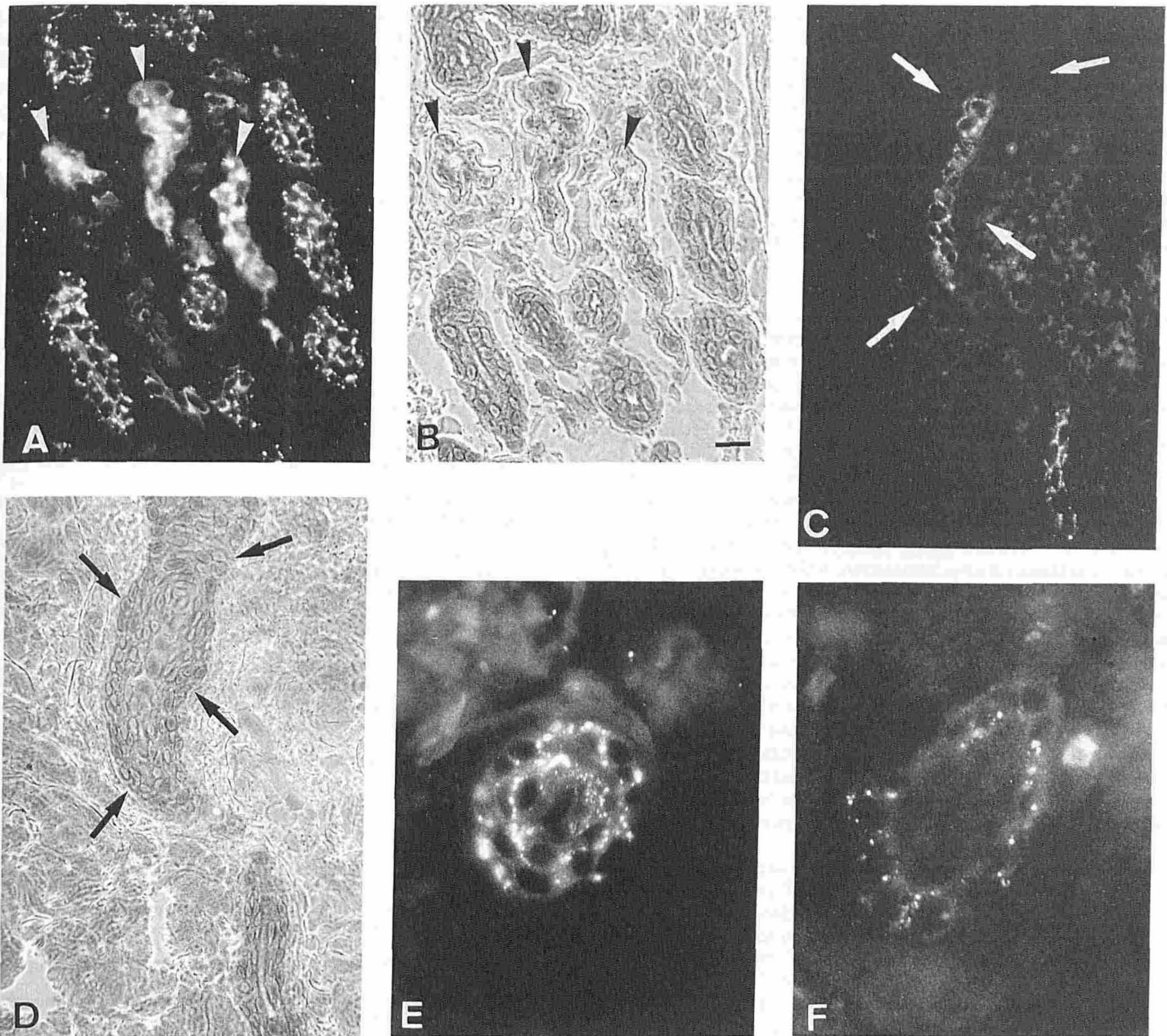

Figure 7. Immunolabeling of eccrine sweat glands for connexins. Incubation of a skin cryosection with polyclonal sera against Cx43 *(F)* and Cx26 *(A–E)*. *A)* The eccrine sweat ducts are abundantly labeled by sera against Cx26. No specific immunostaining was detected on the secretory coils of eccrine glands, which were markedly autofluorescent *(arrowheads)*. *B)* Phase-contrast of the section shown in *A*. *C)* The same antisera against Cx26 immunostained the eccrine sweat ducts in their dermal and intraepidermal portions. In contrast, the interfollicular keratinocytes *(arrows)* surrounding the final portion of the ducts were not stained for Cx26. *D)* Phase-contrast of the section shown in *C*. *E)* High magnification showing the presence of Cx26 immunoreactivity on the two cell layers comprising the wall of an eccrine sweat gland duct. *F)* Immunostaining for Cx43 revealed that, contrary to Cx26, this connexin was predominantly distributed on the basal layer of excretory duct cells. (*Bar,* 30 μm in *A–D* and 12 μm in *E* and *F*.)

Connexins in Dermis Incubation of sections with antibodies against Cx43 resulted in a sparse, punctate immunofluorescent labeling of both papillary and reticular dermis. Northern blots also showed that split human dermis expressed low levels of a 3.0-kb mRNA that was hybridized with a cRNA probe specific for Cx43 (Fig 5). By contrast, no immunolabeling specific for Cx26, Cx32, and Cx40 was detected in the dermal compartment of normal human skin.

DISCUSSION

Using antibodies against different epitopes of Cx and probes that specifically hydridize to their corresponding mRNAs, we have found that two connexins (Cx32 and Cx40) are not expressed in human skin, whereas two others (Cx43 and Cx26) are variably distributed in both its epidermal and dermal compartments.

Cx43 was abundant in the interfollicular epidermis and all epidermal adnexae and was also detected in the dermis, although at much lower levels. The expression of Cx43 by keratinocytes, a cell type that differs markedly in terms of embryologic origin, differentiation program, morphogenetic behavior, and function from other cells expressing Cx43, is another example of the widespread distribution of this gap junction protein [36]. Even though the same antibodies detected Cx43 in different tissues including the epidermis, and Northern blot hybridizations revealed a single transcript encoding this protein in the latter tissue, Western immunoblotting showed that epidermal Cx43 had an apparent molecular weight of about 42 kDa, as in brain but at variance from heart. This difference may be related to a differential phosphorylation level of Cx43, which may be related to the function of gap junction channels [37,38]. It remains to be established whether the Cx43 comprising gap junctions between keratinocytes of skin adnexae is the same as the Cx43 present between interfollicular keratinocytes. The dem-

onstration of dye coupling between follicular keratinocytes indicates that, at least in hair follicles, keratinocyte gap junctions are in the same functional state than in the epidermis [39]. Nevertheless, the presence of Cx43 and dye coupling between keratinocytes from various type of differentiation is not in itself an indication that the gap junction mediated intercellular communication occur without specificity between the epidermis and the different epidermal adnexae, and/or that Cx43 fulfills the same biologic function in these structures.

Immunofluorescence labeling of interfollicular epidermis showed that Cx43 staining is minimal in basal keratinocytes and more marked between the keratinocytes of all spinous [16,17] and granular layers. This differential distribution nicely fits with the variable extent of keratinocyte gap junctions and dye coupling as evaluated by conventional [8] or freeze-fracture electron microscopy [11] and Lucifer Yellow microinjections of human skin [13,40], respectively. Thus, whereas basal keratinocytes have been shown to be connected by few gap junctions and to be infrequently coupled, suprabasal keratinocytes have been found to form much larger and frequent gap junctions and to be extensively coupled to nearby cells. These observations and the lack of detection in the interfollicular epidermis of several other connexins, including Cx26 (see below), indicate that Cx43 could represent one of the predominant if not the sole gap junction protein determining keratinocyte-to-keratinocyte communication in the surface epidermis of human skin.

Furthermore, the striking and abrupt increase in the levels of this protein as keratinocytes leave the basal layer to enter the living, suprabasal layers of the epidermis suggests that a significant quantitative change in Cx43 expression is a novel and major feature in the complex program of keratinocyte differentiation [41,42]. It remains now to be assessed whether the increased expression of Cx43 and of the junctional communication it mediates participates in control of the proliferation and/or differentiation of keratinocytes. Previous studies in other systems have provided evidence for the participation of gap junctional communication in such important cell functions [1,5,43,44].

The second connexin identified in human skin is Cx26, which was found to be abundantly coexpressed with Cx43 in hair follicles, with the notable exception of the sebaceous glands. These findings are in full agreement with the observations in rodent skin, which, however, also showed abundant expression of Cx26 in the interfollicular epidermis ([18], personal observation). The latter finding markedly contrasts with the situation in human skin, where Cx26 is not detected in normal epidermis [16,17]. Our study shows that this difference is actually the reflection of a combined tissue and species specificity, inasmuch as the same antibodies that detected Cx26 in keratinocytes of human skin adnexae (including in the most superficial portion of eccrine sweat duct that crosses the epidermis) and rodent epidermis, failed to do so in keratinocytes of human interfollicular epidermis. The latter tissue is therefore markedly different from that of skin adnexae and from its rodent counterpart not only in terms of morphogenetic development, overall organization, and function, but also with respect to connexin expression.

Cx26 has also been detected in cultures of primary human keratinocytes [45] as well as in reconstituted skin *in vitro* (unpublished data, D. Salomon and A. Limat). Whether this apparent discrepancy with the *in situ* observations can be explained by an adnexal rather than interfollicular origin of the proliferating keratinocytes, or whether, as recently documented for liver cells [46], it reflects a culture-induced alteration in the normal proliferation/differentiation program of interfollicular keratinocytes, remains to be determined.

Eight homologous connexin sequences have been presently identified in the mammalian genome in addition to those of Cx26, Cx32, Cx40, and Cx43 [5,6]. Three of these connexins, Cx31, Cx30.3, and Cx31.1, have been found highly expressed in rodent tail [47,48], the former two (Cx 30.3 and Cx 31.1) have been expressed in keratinocyte cell lines [48], and one of them (Cx31) has been localized in rodent sebocytes [18]. This indicates that skin is a particularly rich source of connexins. It is therefore possible that gap junction proteins other than the four we have investigated here may also be expressed in human skin. Investigations of this possibility will be feasible, with the forthcoming availability of cRNA probes and new antibodies for connexin immunolocalization.

We thank N. K'Bourch, L. Burkhardt, P. Carraux, A. Charollais, S. De Mitri, E. Sutter, and J.-P. Gerber for excellent technical assistance.

This work was supported by grants from the Swiss National Science Foundation (32-30211.90 and 32-34090.92), the Juvenile Diabetes Foundation International (192467), and the Commission of the European Communities (SC1-CT92-0833).*

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