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Original Article

Differential expression of two different homeobox gene families during mouse tegument morphogenesis

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ABSTRACT The expression of six genes belonging to two different homeobox gene families was studied during the embryonic and postnatal morphogenesis of head and body regions of the mouse integument. The first family included the *Otx1* and *Otx2* genes, both related to the *orthodenticle Drosophila* gene and the second was represented by four members of the *Antennapedia* class HOX genes: *Hoxc8* and three *Hoxd* genes, *d9, d11* and *d13*. *In situ* hybridizations with ^{35}S labeled antisense RNA probes were performed on head serial frontonasal sections, as well as entire embryo and postnatal tail longitudinal sections. The expression of these genes shows a differential spatio-temporal pattern along the cephalo-caudal axis. In 12.5-day and 15.5-day embryos, the *Otx2* gene expression is restricted to the nasal epithelium and its associated glands, while the *Otx1* transcripts are present in both nasal and facial integuments, including nasal glands and hair vibrissa follicles. The *Hoxc8* expression first appears in skin at 14.5 days of gestation in the sternal region and is extended at 16.5 days to the thoracic ventral and lumbar dorsal regions. The *Hoxd9* and *Hoxd11* genes are only expressed in the caudal skin from 14.5 days of gestation. The *Hoxd13* transcripts are the last to appear, 2 days after birth, and are limited to the last epidermal cells to differentiate, i.e. those of the hair matrix of the caudal pelage hair follicles. Taken together, these observations strengthen the hypothesis that different homeobox gene families specify the regional identity of the skin in the cephalic and body regions.

KEY WORDS: *gland, hair, homeobox genes, morphogenesis, regionalization, skin*

Introduction

The vertebrate skin is a complex organ formed by the collaboration of two tissues: an epithelium, the epidermis, and a mesenchyme, the dermis. This organ appears to be regionally specified, giving rise to a variety of cutaneous appendages, such as in mouse, pelage hairs, hair vibrissae and exocrine glands. Dermal-epidermal recombination experiments have shown that the regional diversity of skin integument depends mostly on the origin of the dermis, such as the choice between hair vibrissae and hair pelage formation (Dhouailly, 1977), but the molecular basis of skin regionalization remains unresolved.

Homeobox genes have been shown to be critical in patterning the development of many segmental and axial structures in several organisms, including vertebrates (for a review see Holland and Hogan, 1988 or McGinnis and Krumlauf, 1992). These genes were first identified in *Drosophila* by their mutations, which cause homeotic transformations (Lewis, 1978). Eight genes are clustered in two gene complexes: the *Antennapedia* complex and the *Bithorax* complex (Duncan, 1987; Kaufman *et al.*, 1990). In the mouse, as well as in the human, thirty-eight *Antennapedia* class I homeobox genes *(Hox)* are clustered at four genomic loci *(Hoxa* through *Hoxd)* localized on separate chromosomes and showing striking similarities in their organization and expression with the homeotic genes of *Drosophila* (Duboule and Dollé, 1989). These genes share a highly conserved 180-base pair DNA-binding sequence and encode transcription factors which act as morphogenetic regulatory molecules. The role of *Hox* genes in providing positional information has been demonstrated by the effects of loss-of-function and gain-of-function mutations in transgenic mice. For example, the analysis of mice lacking a functional copy of the *Hoxc8* gene revealed anterior transformations of vertebrae (Le Mouellic *et al.*, 1992). The fundamental role of *Hox* genes, particu-

Abbreviations used in this paper: Antp, Antennapedia; d, day; HOX, antennapedia class homeobox gene; PBS, phosphate-buffered saline.

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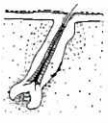
TABLE 1

COMPARISON OF THE MAIN STAGES OF HAIR VIBRISSA, HAIR PELAGE AND NASAL GLAND DEVELOPMENT

STAGE	hair vibrissae	dorsal pelage hairs	caudal pelage hairs	nasal glands
0	 10.5 d*	12.5 d	16.5 d	 12.5 d
1	 12.5 d	14.5 d	17.5 d	 13.5 d
3	 13.5 d	16.5 d	18.5 d	 14.5 d
7	 15.5 d	at birth	2 pnd**	 15.5 d
8	 17.5 d	2 pnd	4 pnd	 16.5 d

* d= days of gestation
** pnd= postnatal days

larly those of the *Hoxd* locus, in patterning the development of the limb and of the body axial structures is also well documented (Duboule, 1992). Conversely, two other homeobox-containing gene classes have been recently isolated in mice and shown to have restricted expression areas in the developing brain: the *Otx1* and *Otx2* genes, related to the *orthodenticle Drosophila* gene (Simeone *et al.*, 1992a), and the *Emx1* and *Emx2* genes, related to the *empty spiracle Drosophila* gene (Simeone *et al.*, 1992b).

Although expression of Hox genes in human, murine and chicken embryonic skin has been reported, little is known about their spatial and temporal distribution in the cutaneous appendages. The expressions of two homeobox-containing genes, namely *Hoxc1* and *Hoxc8*, have been reported during human fetal skin development (Simeone *et al.*, 1987). The expression of genes of the *Hoxb* locus has been detected in fetal and adult murine skin by RNase protection and reverse transcription-polymerase chain reaction (RT-PCR) analysis from day 12 to day 18 of gestation (Detmer *et al.*, 1993). Likewise in chick, *Hoxc6* and *Hoxd4* display a gradient along the antero-posterior axis of dorsal feather buds (Chuong *et al.*, 1993).

In the present study, we investigate the hypothesis that two different homeobox gene classes, *Otx* and *Hox*, identify respectively the skin of anterior cephalic and body regions in mouse. The expression distribution of six genes, namely *Otx1, Otx2, Hoxc8, Hoxd9, d11* and *d13*, was studied using *in situ* hybridization with radiolabeled antisense RNA probes during mouse skin embryonic and postnatal morphogenesis. The results reveal a spatio-temporal expression: *Otx2* and *Otx1* transcripts appear first and are restricted respectively to the nasal and facial integument, whereas *Hoxc8, Hoxd9 and d11* transcripts appear later and identify respectively the body and caudal skin. Moreover, the *Hoxd13* gene is the last to be transcribed and its transcripts are limited to the last keratinocytes to differentiate, forming the hair matrix of the pelage hair follicles of the tail.

Results

Mouse tegument morphogenesis

The facial integument differentiates at between 11.5 and 15.5 days of gestation, while the body and caudal skin morphogenesis begins respectively by 14.5 and 16.5 days of gestation. The developmental steps are similar for vibrissa and pelage hair follicles, and the staging has been established by Hardy (1968) and extended by us to the caudal hair follicles (Table 1). The initial hair bud (stage 1) corresponds to a placodal thickening of the basal epidermal layer associated to a dermal condensation. The placodal epidermal cells then proliferate to form the hair peg (stages 2 to 3), which is associated to a round dermal papilla. Hair bud keratinocytes differentiate into seven concentric layers which form the hair follicle, beginning at stage 4 and ending at stage 8 with the emergence of the hair shaft. The first steps of nasal gland formation are similar, but the nasal peg is not associated to a dermal papilla (Viallet and Dhouailly, 1994). Its differentiation into a glomerular eccrine gland starts with the appearance of a lumen by stage 4 and the growing of its tip, which forms the glomerulus by stage 8 (Table 1).

Spatial distribution of Otx1 and Otx2 transcripts and morphogenesis of the facial integument

The expression pattern of the *Otx1* and *Otx2* genes was studied in 12.5- to 18.5-day embryos.

The *Otx1* gene was expressed by 12.5 days of gestation in the

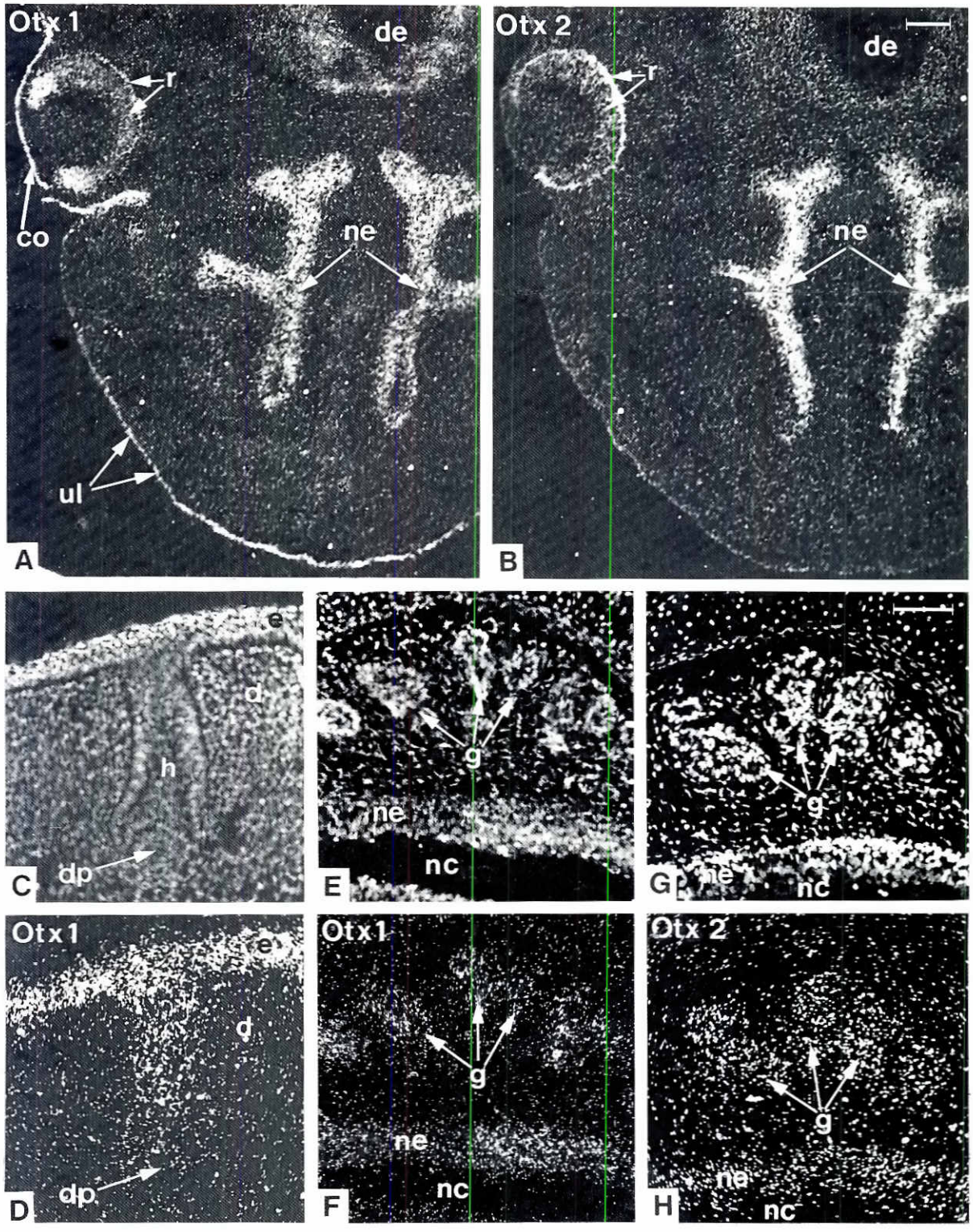


Fig. 1. Distribution of *Otx1* and *Otx2* transcripts in the facial epithelia in fronto-nasal head sections of mouse embryos. **(A)** *At 12.5 days of gestation, the Otx1 transcripts are present in both neurodermal and ectodermal epithelia, including diencephalon (de), pigmented and nervous retina (r), corneal epithelium (co), nasal epithelium (ne) and upperlip epidermis (ul).* **(B)** *The next section, labeled with the Otx2 probe, shows a transcript distribution restricted to the diencephalon, retinas and nasal epithelium.* **(C-F)** *Distribution of the Otx1 transcripts at 15.5-days of gestation in the upperlip epidermis (e) and its associated hair vibrissa follicles (h) as well as in nasal epithelium (ne) and associated nasal glands (g).* **(G and H)** *Distribution of the Otx2 transcripts in the nasal epithelial cells. dp, dermal papilla; nc, nasal cavity.* **(A,B,D,F,H)** *Darkfield illumination.* **(C,E and G)** *Propidium iodide staining. Bars, 200 μm* **(A,B)**; *50 μm* **(C-H).**

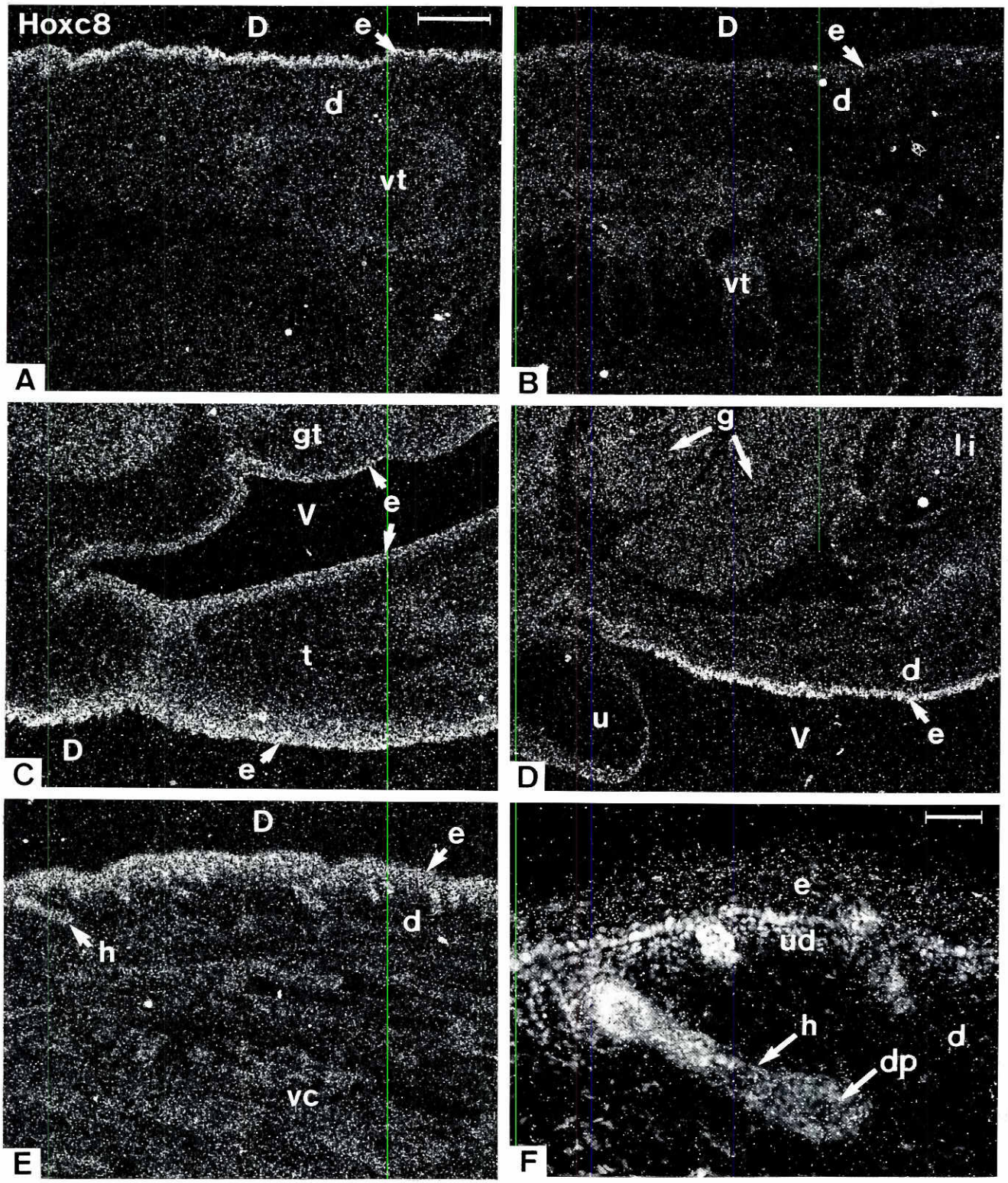


Fig. 2. Skin distribution of *Hoxc8* transcripts in longitudinal sections of 16.5- and 18.5-day mouse embryos. **(A-D)** *16.5 days of gestation. The Hoxc8 signal is present in the dorsal epidermal cells* **(A)** *and absent in the dorsal thoracic skin* **(B)**. *The dorsal signal persists in the dorsal caudal region* **(C)** *and reaches its highest level in the thoracic ventral epidermis* **(D)**. **(E-F)** *18.5 days of gestation. In the dorsal region, the hair follicles (h) (stage 3 to 6), the epidermis* **(E)** *and their associated dermal cells* **(F)** *are labeled. D, dorsal; V, ventral; d, dermis; dp, dermal papilla; e, epidermis; g, gut; gt, genital tubercle; li, liver; t, tail; u, umbilical cord; ud, upper dermis; vc, vertebral column; vt, vertebrae.* **(A-E)** *Darkfield illumination.* **(F)** *Both darkfield and fluorescent illumination on propidium iodide staining. Bars, 400 μm* **(A-E)**; *50 μm* **(F)**.

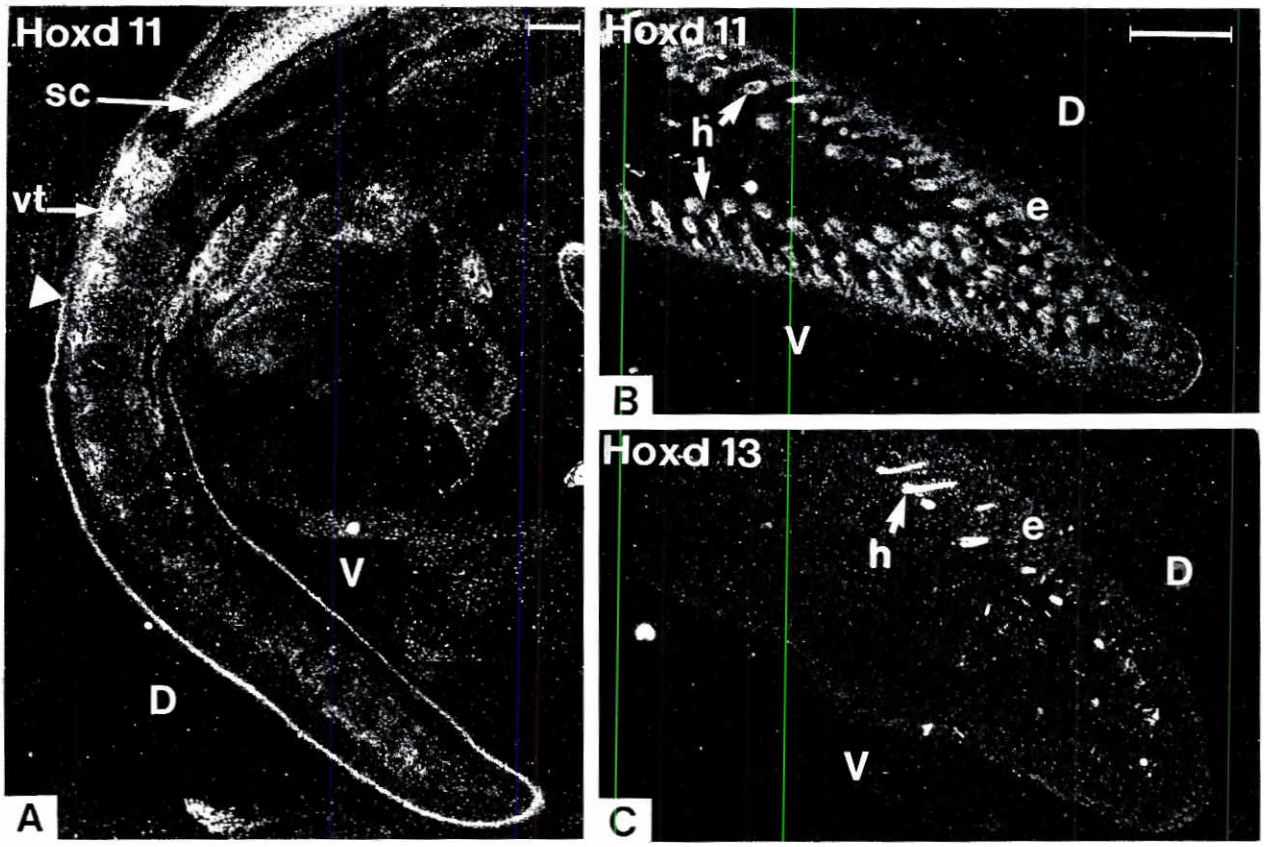


Fig. 3. Distribution of *Hoxd11* and *Hoxd13* transcripts in longitudinal sections of caudal skin of 16.5-day mouse embryo (A) and of 2-day-old mouse offspring (B and C). **(A)** *The anterior limit of expression of the Hoxd11 gene coincides with the base of the tail (arrow head). Note that this skin expression is more posterior than in vertebrae (vt) and spinal cord (sc).* **(B)** *Two days after birth, the Hox d11 transcripts are limited to the basal layer of the epidermis (e) and to the differentiating hair follicles (h).* **(C)** *At the same stage, the Hoxd13 transcripts appear for the first time in the matrix of the hair follicles (h). D, dorsal; V, ventral.* **(A-C)** *Darkfield illumination. Bar, 400 μm.*

neuroderm forming the brain and the retinas as well as in the facial epithelial cells, including nasal and corneal epithelia and upper-lip epidermis (Fig. 1A). In contrast, the *Otx2* expression domain was restricted to the neurodermal cells and the nasal epithelium (Fig. 1B). By 15.5 days of gestation, this expression pattern was conserved and extended to the different facial appendages: the *Otx1* transcripts were localized both in the epithelial cells forming the hair vibrissa follicles (Fig. 1C,D) and the nasal glands (Fig. 1E,F), whereas the *Otx2* transcripts were only found in the distal and proximal nasal epithelia and their associated glands (Fig. 1G,H). No significant expression of these two genes was observed in the dorsal or caudal regions of the skin at any of the studied developmental stages.

Spatial distribution of Hoxc8 transcripts and body skin morphogenesis

The distribution of the *Hoxc8* transcripts was studied during body hair pelage formation (14.5-, 16.5- and 18.5-day embryos). They were first detectable in the ventral thoracic skin at 14.5 days of gestation (stage 0, data not shown). By 16.5 days of gestation (stage 1 to 3 of pelage hair follicle), the *Hoxc8* gene is expressed according to two graded patterns. Along the cephalo-caudal axis its expression is restricted in the dorsal region to the lumbar (Fig. 2A) and caudal skin (Fig. 2C), while it is more extended in the ventral region (Fig. 2D), from the throat to the caudal tip. Transcripts were never found in the head or in the thoracic dorsal skin (Fig. 2B). Two days later, the distribution of the *Hoxc8* expression was similar. It should be noted that the transcripts were found not only in the epidermis and epithelial cells of the hair follicles, but also in the fibroblasts of the dermal papilla as well as of the upper dermis (Fig. 2E,F), in lumbar, ventral, and caudal skin.

Spatial distribution of Hoxd9, d11 and d13 transcripts and caudal skin morphogenesis

On serial longitudinal embryo sections at days 14.5, 16.5 and 18.5 of gestation, and longitudinal tail sections from newborn, 2- and 4-day-old offspring, the expression of *Hoxd9, d11* and *d13* was restricted to the most posterior part of the embryo, i.e. the caudal region. No signal was found in other skin regions, including the lumbar one. Note that the pelage hair follicles of the tail develop much later than those of the dorsal region, although we focused our observations on the dorsal tail skin, which differentiates slightly earlier than that of the ventral side.

During the first stages of caudal skin morphogenesis, transcripts of the *Hoxd9* and *d11* (Fig. 3A) genes appeared in the epidermal skin component, and were still expressed two days after birth (Fig. 2B). In contrast, the Hoxd13 transcripts were only found at the last stage (Fig. 3C). These three genes showed the same

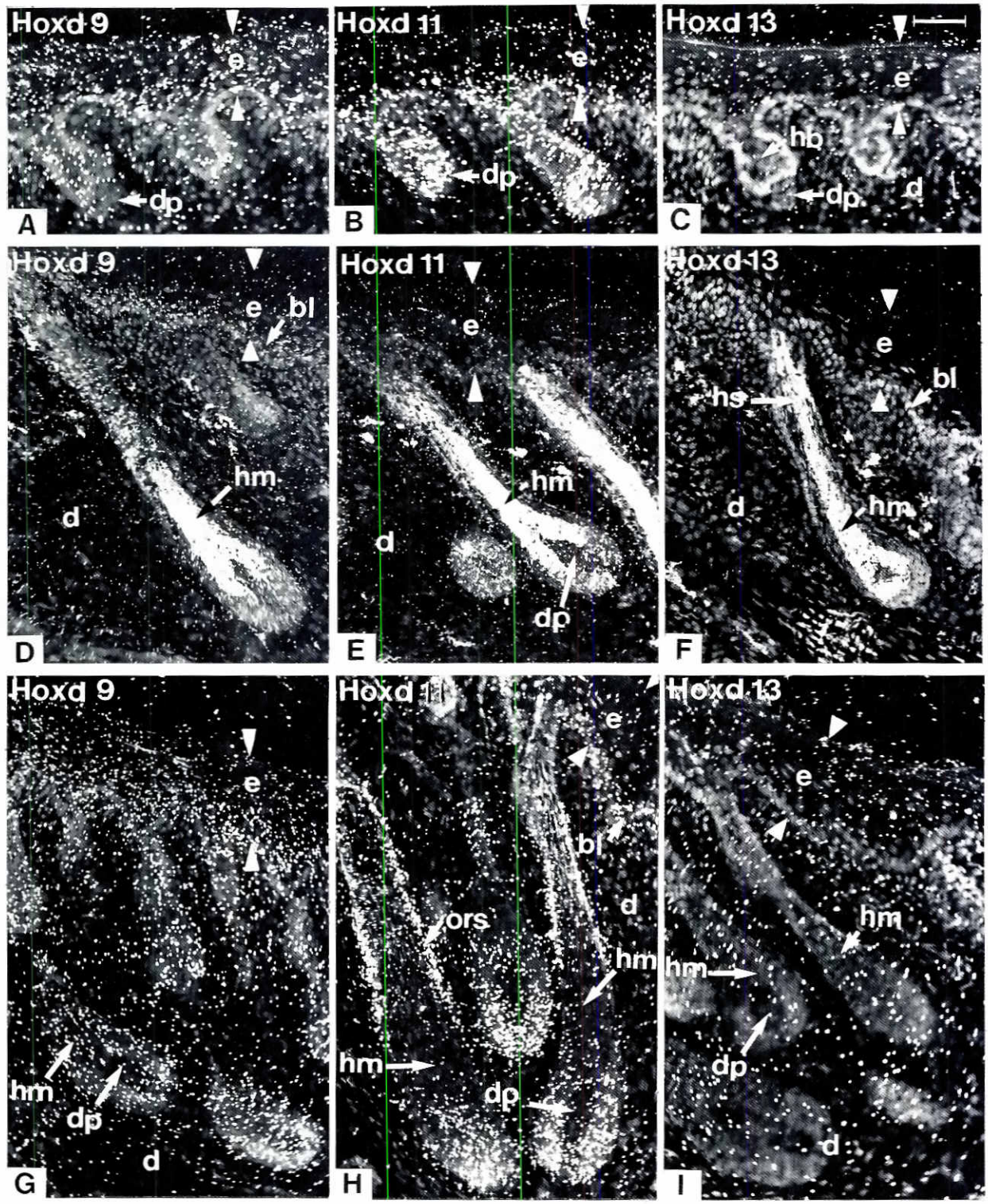


Fig. 4. Distribution of *Hoxd9, Hoxd11* and *Hoxd13* transcripts at three different stages of hair follicle morphogenesis in longitudinal sections of mouse tail. **(A-C)** *Stage 3 hair buds of 18.5-day embryo. The Hoxd9* **(A)** *and Hoxd11* **(B)** *genes are expressed in all the strata of the epidermis as well as in the hair epithelial peg. There is no signal with the Hoxd13 probe* **(C)**. **(D-F)** *Stage 7 hair follicles, 2 days after birth. The Hoxd9* **(D)** *and Hoxd11* **(E)** *genes are expressed in the basal layer (bl) of the epidermis as well as in the hair matrix (hm). In contrast the Hoxd13 transcripts* **(F)** *are limited to the hair matrix.* **(G-I)** *Stage 8 hair follicles of 4-day-old offspring. A slight signal is still observed with the Hoxd9 and Hoxd11 probes* **(G,H)**, *while the Hoxd13 signal* **(I)** *has totally disappeared. d, dermis; e, epidermis; hb, hair bud; ors, outer-root sheath.* **(A-I)** *Both darkfield and fluorescent illumination on propidium iodide staining. Bar, 50 μm.*

anterior expression limit at the base of the tail. This expression was posterior to that observed for the same genes in the spinal cord and the somites (Fig. 3A). The *Hoxd9* and *d11* signal both appeared in the epidermis at day 14.5 (stage 0 of hair formation in caudal skin) and reached their highest level by day 16.5 (stage 2 of hair formation in caudal skin). At day 18.5 of gestation (stage 3 of hair formation), the *Hoxd9* signal (Fig. 4A) appeared uniformly distributed in the epidermis and the hair bud epithelial cells, whereas the *Hoxd11* signal is preferentially localized in the basal epidermal layer and the hair epithelial downgrowth (Fig. 4B). By these three early stages (0 to 3) of the caudal first hair cycle, the *Hoxd13* signal was never observed (Fig. 4C).

Two days after birth, the caudal hair follicles reached stage 6. The hair cells or trichocytes, which develop from the matrix and contribute to the forming hair shaft, are arranged in a basally concave cone. They expressed simultaneously the three studied *Hoxd* genes (Fig. 4D-F). The *Hoxd9* and *Hoxd11* transcripts were present in the basal epidermal layer and in all the epithelial follicular cells, whereas the *Hoxd13* transcripts were confined to the hair matrix epithelial cells. Four days after birth, the *Hoxd9* and particularly the *Hoxd11* genes were still expressed in the uppermost outer root sheath of stage 8 follicles and in the epithelial cells surrounding the dermal papilla (Fig. 4G,H), whereas their expression in the hair matrix cells disappeared. In addition, significant *Hoxd13* expression was not observed afterwards (Fig. 4I).

It should be noted that the signals corresponding to the three *Hoxd* genes were never higher than the background level in the dermis and more precisely the dermal papilla cells, for all the studied stages.

Discussion

Using *in situ* hybridization, we analyzed the differential expression of six genes belonging to the homeobox gene family in the skin along the antero-posterior axis of mouse embryos and postnatal offspring. Each probe hybridized specifically to its complementary mRNA as shown by their distinct labeling pattern at each developmental stage.

Each of the different homeobox genes studied revealed a clearly restricted expression pattern in the skin over the period of its organogenesis. The two homeobox genes related to the *orthodenticle* gene of the *Drosophila*, *Otx1* and *Otx2*, already shown to play a crucial role in the development of the brain (Simeone *et al.*, 1992a), were exclusively expressed in the cephalic epithelia. Both the upper lip and nasal epithelial cells expressed the *Otx1* gene at the onset of their organogenesis, while the *Otx2* transcripts were limited to the nasal epithelium. These two *Otx* expression domains, which overlap only in the nasal epithelium, persisted during the development of cutaneous appendages such as nasal glands and hair vibrissae follicles. Regarding the *Hoxc8* gene, our results are consistent with the graded pattern of its expression in adult murine dorsal skin, as described by Bieberich *et al.* (1991), who used RT-PCR amplification and transgenic mice to analyze its distribution. In mouse embryo, the *Hoxc8* gene was expressed according to two graded patterns along the cephalo-caudal and dorso-ventral axes. Its stronger activity was observed in ventral skin and to a lesser degree in lumbar and caudal skin. Its expression was not restricted to the keratinocytes from the epidermis and hair follicles, but extended to their closely associated dermal cells (dermal papilla and upper dermis). It should be noted that the *Hoxc8* gene is the only one among those presently studied to be expressed both in the epidermal and dermal components of the skin, and particularly in the dermal papilla. An intriguing unpublished observation was reported by one of us (Le Mouellic): a null mutation in the *Hoxc8* gene induces two glabrous zones in the lumbar region, symmetrically distributed on each side of the dorsal rostro-caudal axis. Thus it could be suggested that the *Hoxc8* expression in the papilla cells may be linked to their well-known hair inductive capacity (Jahoda *et al.*, 1993). The embryonic and fetal development of the caudal skin and of its appendages is accompanied by a series of changes in the expression of the three studied *Hoxd* genes. The *Hoxd9* and *Hoxd11* genes were first detected in the caudal epidermis at the onset of its differentiation. Then their expression remains linked to the proliferative compartment of epidermis and hair follicles, namely the epidermal basal layer and the hair matrix, the latter giving rise to the hair shaft. At the end of the first hair cycle, the *Hoxd9* and *Hoxd11* genes are no longer expressed in the hair matrix. The *Hoxd13* transcripts appear for the first time in the last epidermal cells to differentiate, i.e. those of the matrix of the stage 7 caudal hair follicles, and disappear when hair morphogenesis is accomplished by stage 8.

Thus, as was previously established for the vertebral column and the limb (for a review see Duboule, 1992), the *Hox* genes appear to be expressed in a spatially and temporally restricted manner in skin, suggesting that the colinearity model might also be implicated in the development of this organ. Another intriguing fact is that the expression of the *Hoxd9* and *d11* genes, already shown to be more posterior in the somites than in the spinal cord (Duboule, 1992), is still more posterior in the epidermis than in the somites, since they are restricted to the caudal integument. Furthermore, at all the stages studied, the *Hoxd* transcripts are surprisingly limited to the epidermal component of the skin. This last result may imply that somitic cells of the dermatome, which are known to give rise to the dermis (Mauger, 1972), lose their ability to express these genes while migrating. The differential distribution of *Otx* and *Hox* genes in head and body epithelial cells may be correlated to their different embryonic origins. Classical studies in chick embryo (Couly and Le Douarin, 1988; Noden, 1988), and recent data in mouse (Osumi-Yamashita *et al.*, 1994) have established the crucial role of cranial neural crest migration in the formation of facial organs. Now, the *Otx* genes are expressed in the developing rostral brain (Boncinelli *et al.*, 1993), whereas the most anterior point at which the *Hox* network acts in the central nervous system is located at the anterior part of the hindbrain (for a review see Hunt and Krumlauf, 1992). Our results show that *Otx* genes expression is confined to the head epithelia, but further studies involving the expression of 3'*Hox* genes are required to specify the anterior boundary of the *Hox* genes in the skin. Different epithelial transdifferential abilities appear linked to this homeobox genes distribution: when treated with retinoic acid only the mouse epidermal cells which express the *Otx1* gene, namely the upperlip epidermal cells, are able to form eccrine glands instead of hair follicles; in the same conditions, the mouse dorsal epidermis does not undergo a glandular metaplasia (Viallet and Dhouailly, 1994).

Taken together, these results show that homeobox genes are not only expressed during early embryonic development but also participate in later developmental processes such as skin morphogenesis. The present observations strengthen the hypothesis that different homeobox gene families may play a significant role in specifying the regional and spatial identity of

the skin. Further analysis of other genes belonging to these families will allow the establishment of the Hox code implicated in skin appendage regionalization.

Materials and Methods

Sample preparation

Mice of the OF1 Swiss strain were mated and the day of the vaginal plug was counted as 0.5. Embryonic and postnatal samples were embedded in Tissue Teck® Serial 10 µm sections were collected on aminoalkyl-silane treated slides. Frontonasal sections were performed on heads of 12.5-, 13.5-, and 15.5-day embryos. Longitudinal sections involved 14.5-, 16.5- and 18.5-day embryos and only the tails from newborn, 2- and 4-day-old offspring. Slides were air dried and stored at -70°C before hybridization. The developmental stages were chosen according to the steps of cutaneous appendage morphogenesis in the various skin regions, as defined by Hardy (1968), Viallet and Dhouailly (1994) and from our unpublished data on caudal skin (Table 1).

In situ hybridization

In situ hybridization on sections was carried out essentially as described by Décimo *et al.* (1994). ^{35}S-labeled antisense RNA probes (specific activity of $5x10^8$ $cpm^{-1}.mg^{-1}$) were synthesized using either T7 or T3 polymerase *in vitro* transcription reactions with full length mouse cDNAs coding for *Otx1*, *Otx2*, *Hoxc8*, *Hoxd9*, *Hoxd11* and *Hoxd13*. Slides were overlaid with 50 µl of hybridization solution containing the heat-denatured radioactive probe (20,000 cpm/µl) and hybridized overnight at 50-52°C. Slides were then washed under stringent conditions and treated with RNase A (20 mg/ml) and RNase T1 (1 U/ml) (Boehringer), for 30 min at 37°C, in order to remove unhybridized and nonspecifically bound probes. Autoradiography was performed with Kodak NTB-2 nuclear track emulsion. Exposure times were between 2 and 5 weeks. After developing, sections were stained with a DNA fluorochrome (propidium iodide, 10 $mg.ml^{-1}$) and mounted in Surgipath®. Sections were analyzed and photographed with an Olympus BH-2 microscope using dark-field illumination.

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