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Original Article

Colinearity and non-colinearity in the expression of *Hox* genes in developing chick skin

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ABSTRACT ***Hox*** **genes are usually expressed temporally and spatially in a colinear manner with respect to their positions in the** ***Hox*** **complex. We found that these characteristics apply to several** ***Hox*** **genes expressed in developing chick skin** ***(Hoxb-4, Hoxa-7*** **and** ***Hoxc-8),*** **and we classed this group of genes as regionally restricted. To our surprise, we found that most of the** ***Hox*** **genes we examined are regionally unrestricted in their expression in the embryonic chick skin. This second group includes the** ***Hoxd*** **genes,** ***Hoxd-4*** **to** ***Hoxd-13, Hoxa-11*** **and** ***Hoxc-6*****. Temporally, the expression of the regionally restricted genes can be observed by E5 within the epidermis, whereas the spatially unrestricted genes are not expressed in the epidermis until E6.25. Unexpectedly, we found that all the unrestricted genes are expressed concomitantly and therefore do not conform to temporal colinearity. Moreover, the dermal expression for both groups occurs later, but maintains the same anteroposterior patterning to that seen previously in the epidermis. During embryonic day 7-8, expression for all genes is up-regulated within the dense dermis whilst being reduced within the inter-bud regions. Later expression within the bud mesenchyme is down-regulated whilst high levels of transcriptional activity are detectable within the epidermal sheath of each feather bud. These results indicate that the transcriptional activity of** ***Hox*** **genes in the developing chick skin could be important during embryonic skin patterning both by providing regionally restricted positional cues, and also by imparting generic signals necessary for feather morphology.**

KEY WORDS: *Hox genes, expression, chick, skin, colinearity*

Introduction

Most tetrapod vertebrates possess 39 *Hox* genes, organized in four related clusters. These clusters lie on four separate chromosomes, and each is related to the clustered homeotic genes of *Drosophila* and other animals. *Hox* genes encode DNA binding transcription factors that regulate their own transcription, the transcription of other *Hox* genes or the transcription of other downstream effector genes (de la Cruz *et al*., 1999 and references therein; reviewed in Krumlauf, 1994). Based on DNA sequence similarities and on the position of the genes on their respective chromosomes, individual members of the four linkage groups have been classified into thirteen paralogous families (Scott, 1992). *Hox* genes provide patterning information for A-P axis specification within the spinal cord (Tiret *et al*., 1998), hindbrain (Goddard *et al*., 1996), branchial arches (Vieille-Grosjean *et al*., 1997), paraxial (somitic) mesoderm and its derivatives (Burke *et al*., 1995; Favier *et al*., 1996; Fromental-Ramain *et al*., 1996) and the lateral plate mesoderm and its derivatives (Nowicki and Burke, 2000). In these structures the various *Hox* genes are expressed in discrete spatial domains that overlap posteriorly, but which extend to different anterior limits along the body. These anterior boundaries are colinear with their relative chromosomal position (Duboule and Dolle, 1989). This property, know as structural colinearity, reflects their primary role in specifying regional identity to body segments along the A-P axis as each topographical zone expresses a unique combination of *Hox* genes.

Vertebrate skin is composed of two distinct cell types: epithelial which forms the epidermis, and mesenchymal which forms the dermis. Experiments in chick have shown that there are regional differences in the cellular origins of the dermis. In the head the dermis mostly differentiates from neural crest cells (Le Lievre and Le Douarin, 1974, 1975; Couly *et al*., 1993). In the trunk the lateral and ventral dermis originate from the somatopleure (Murray, 1928; Mauger, 1972), whilst the dorsolateral dermis derives from cells originating in the dermamyotomes of the somites (Mauger and Sengel, 1970; Mauger, 1972; Christ *et al*., 1983).

Recent advances in determining the molecular basis of skin development has highlighted members of the *Hox* family of

Abbreviations used in this paper: A-P, anteroposterior.

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homeobox genes. Kanzler *et al.* (1997) reported that by E6.5 transcripts of *Hoxc-8* are detectable in a regionally restricted manner within both the epidermis and dermis of the chick embryo. This expression has a sharp anterior boundary at the level of the fifth thoracic vertebra, which corresponds with the limit observed earlier within the somites. A simple explanation for this would be that *Hox* expression domains are established in dermal precursor cells before they leave the somite, and that these are then simply maintained as the cells colonize the dorsal dermal territories. So far, however, there does not seem to be published evidence for this. This observation upon the expression of *Hoxc-8* in skin appears to be entirely consistent with the general rule that *Hox* genes are expressed along the A-P axis of the embryo with structural colinearity.

Surprisingly, however, the expression of mouse *Hoxc-13* in skin contravenes this basic rule (Duboule, 1998a; Godwin and Capecchi, 1998; Godwin and Capecchi, 1999). *Hoxc-13*, being a 5'-located gene, is expressed in neural tube and prevertebrae only in posterior parts of the body. Yet in hair follicles, *Hoxc-13* is expressed along the entire length of the body, including the vibrissae and the tongue. The knockout of *Hoxc-13* produces an overall alopecia due to shedding of structurally weakened hairs (Godwin and Capecchi, 1998).

The objective in the present work was to investigate the expression patterns in developing skin for a variety of different chick *Hox* genes. We wanted to test whether they followed the A-P distribution of chick *Hoxc-8* (spatially restricted) or of the mouse *Hoxc-13* (spatially unrestricted). To investigate the putative involvement of *Hox* genes in providing positional information to skin cells a series of *in situ* hybridization experiments were performed. The experiments show that during skin development *Hox* genes are expressed in two different profiles. One group of genes (*Hoxa-7*, *Hoxb-4* and *Hoxc-8*) are expressed in skin in a manner that is colinear with the pattern of expression within somites. These genes exhibit sharp anterior boundaries of expression within both the neural tube and the dorsal skin. The second group, which includes most *Hoxd* genes as well as *Hoxa-11* and *Hoxc-6*, are expressed ubiquitously within the skin from the tail to the head, although they maintain sharp anterior boundaries of expression within the neural tube.

The results therefore suggest that patterning of the chick skin by *Hox* genes is complex and that two distinct expression profiles are utilized (regionally restricted and regionally unrestricted). The functions of these genes in determining skin phenotype awaits disclosure, but it would seem that spatial colinearity does not govern all *Hox* gene expression in late onset developmental programs.

Results

We examined the expression patterns in developing skin for a variety of different chick *Hox* genes to test whether they followed the A-P distribution of chick *Hoxc-8* (spatially restricted) or of mouse *Hoxc-13* (spatially unrestricted). Radioactive *in situ* hybridization experiments were performed on sections of E3-E12 chick embryos. The probes used are described in Materials and Methods. Derivation of the *Hoxd* probes required restriction enzyme mapping within the chick *Hoxd* cluster, and the results of this are shown in Figure 1. As for mouse and human, *Hoxd-5*, *Hoxd-6* and *Hoxd–7* were apparently missing, since no hybridization of homeobox probes was detected to this region.

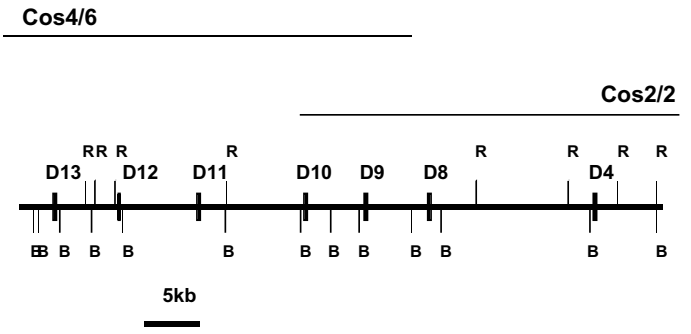


Fig. 1. Map of cosmids 4/6 and 2/2 showing restriction enzyme sites and locations of *HoxD* genes. *B, BamH1; R, EcoR1.*

Some Hox *Genes are Regionally Restricted in their Expression within the Embryonic Chick Skin*

Our initial findings on E7 chick embryos confirmed the published results for *Hoxc-8* (Kanzler *et al.*, 1997). Thus, *Hoxc-8* was seen to be expressed within prevertebrae up to the level of thoracic prevertebra 5 and, similarly, expression within dorsal skin extends from the posterior of the embryo up to the level of thoracic prevertebra 5 (Fig. 2A, arrow). Spatial restriction within the dorsal skin was also observed for *Hoxb-4* (Fig. 2B) and *Hoxa-7* (Fig. 2C). Expression within the skin is more anterior for *Hoxb-4* (cervical prevertebra 4-5) than for *Hoxa-7* (cervical prevertebra 9). Overall, therefore, the expression of these three *Hox* genes in dorsal skin apparently conforms to the rule of structural colinearity, with correspondence between the ordering of their expression domains along the embryo and the ordering of the genes along their clusters. Expression patterns of *Hoxb-4*, *Hoxa-7* and *Hoxc-8* within hindbrain, spinal cord, prevertebrae, spinal ganglia, and intestine are all as described in earlier publications (Tiret *et al.*, 1998; Folberg *et al.*, 1999; Gaunt *et al.*, 1999; Pitera *et al.*, 1999; Beck *et al.*, 2000; Gaunt, 2000).

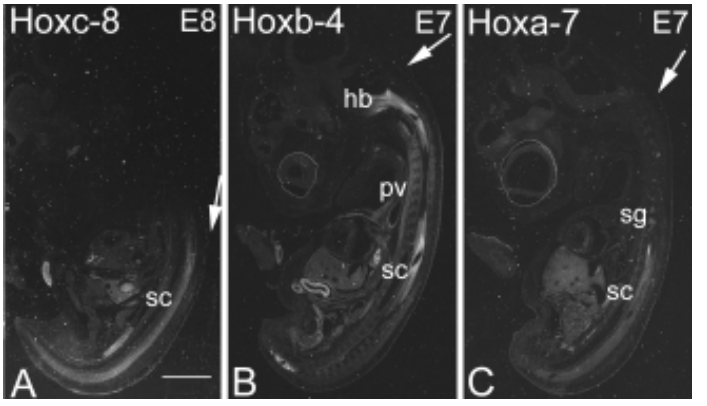


Fig. 2. Some *Hox* genes are regionally restricted in their expression within the developing chick skin. In situ *hybridization of sagittal sections with anti-sense probes.* **(A)** Hoxc-8; **(B)** Hoxb-4; **(C)** Hoxa-7. *The position of the* Hoxc-8 *expression boundary (arrow in A) lies at approximately the same level as in the adjacent prevertebrae (thoracic prevertebra 5). Similarly, the* Hoxb-4 *expression boundary in skin (arrow in B) lies at the same level as in prevertebrae (cervical prevertebra 4/5). For* Hoxa-7, *the anterior boundary in skin (arrow in C) lies between that of* Hoxb-4 *and* Hoxc-8. *sc, spinal cord; hb, hindbrain; sg, spinal ganglia; pv, prevertebrae. Darkfield illumination. Scale bar, 2.0 mm.*

Hoxd *Genes and some Paralogues are Regionally Unrestricted in their Expression within the Embryonic Chick Skin*

Hoxd gene (*Hoxd-4*, *Hoxd-8*, *Hoxd-9*, *Hoxd-10*, *Hoxd-11 Hoxd-12* and *Hoxd-13*) expression was examined in embryos ranging from embryonic day 6 to 12 (E6-E12). As expected, and in accordance with structural colinearity, the expression domains within neural tube and pre-vertebrae were found to correspond with the position of genes along the cluster. In contrast, however, expression of all the genes within the skin was regionally unrestricted, extending from posterior regions (tail) to very anterior domains (head) (Fig. 3 A-H). We considered that this expression profile might have been accounted for by cross-reaction of the homeobox. To eliminate this doubt we did a series of *in situ* hybridizations using probes of *Hoxd-10* and *Hoxd-11* in which the homeobox region had been removed. The results were similar to those obtained with the respective full-length probes (not shown). Expression of *Hoxd-11* and some other *Hoxd* genes was even evident in the epithelial cells of the cornea as well as the tongue, beak and the egg tooth (Fig. 3H, and not shown).

Our study was widened to investigate expression profiles of additional *Hox* genes from other clusters. *Hoxc-6* (Fig. 3I) transcription was detected within neural tube up to the level of the spinal cord/hindbrain junction. Expression is also evident within the hindlimb. Expression of *Hoxa-11* (Fig. 3J) is seen within the prevertebrae up to the level of lumbar prevertebra 5. Similarly to the *Hoxd* genes, *Hoxc-6* and *Hoxa-11* are expressed ubiquitously within the skin (Fig. 3I and 3J). Expression extends from the posterior of the embryo to the head skin territories.

Expressions of *Hoxd-11*, *Hoxa-11*, *Hoxd-12* and *Hoxd-13* in the uteric duct (Fig. 3E, 3J and not shown); *Hoxd-4*, *Hoxd-8*, *Hoxd-12* and *Hoxd-13* in the mesonephros (Figs. 3F, 3G and not shown); and *Hoxa-11*, *Hoxd-12* and *Hoxd-13* in the rectum and genital tubercle (Fig. 3F and not shown), were all as described in earlier publications (Peichel *et al.*, 1997; Potter and Branford, 1998; Beck *et al.*, 2000).

Hox *Gene Expression within the Embryonic Chick Skin is Temporally Dynamic*

For the *Hox* genes that show a regionally restricted expression profile (*Hoxc-8*, *Hoxa-7*, *Hoxb-4*), expression within the skin-forming region is dynamic. Initial expression is observed within the epidermis. This occurs by embryonic day 5 (Fig. 4A and not shown). Epidermal expression is maintained as dermal expression commences approximately 1.5 days later, at E6.5 (Fig. 4B, 4C and 4D). Furthermore, the expression within the dermis exactly mimics the epidermal expression in that the anterior boundaries are the same.

We next examined temporal regulation of those *Hox* genes that are regionally unrestricted in their skin expression. We found that expression of *Hoxd* genes was not detectable in the skin up to six days of development (Figs. 4E and 4F and not shown), but could be detected ubiquitously over the skin after 6.25 days. This initial expression was confined to the epidermis, but soon afterwards, by about 6.5 days, there was also expression within the dermis (Fig. 4G). Thus the expression of both the regionally restricted and regionally unrestricted genes is initiated in the epidermis in a temporally dichotomous manner followed by a synchronous up-regulation in the dermis at E6.5. Expression within the dermis

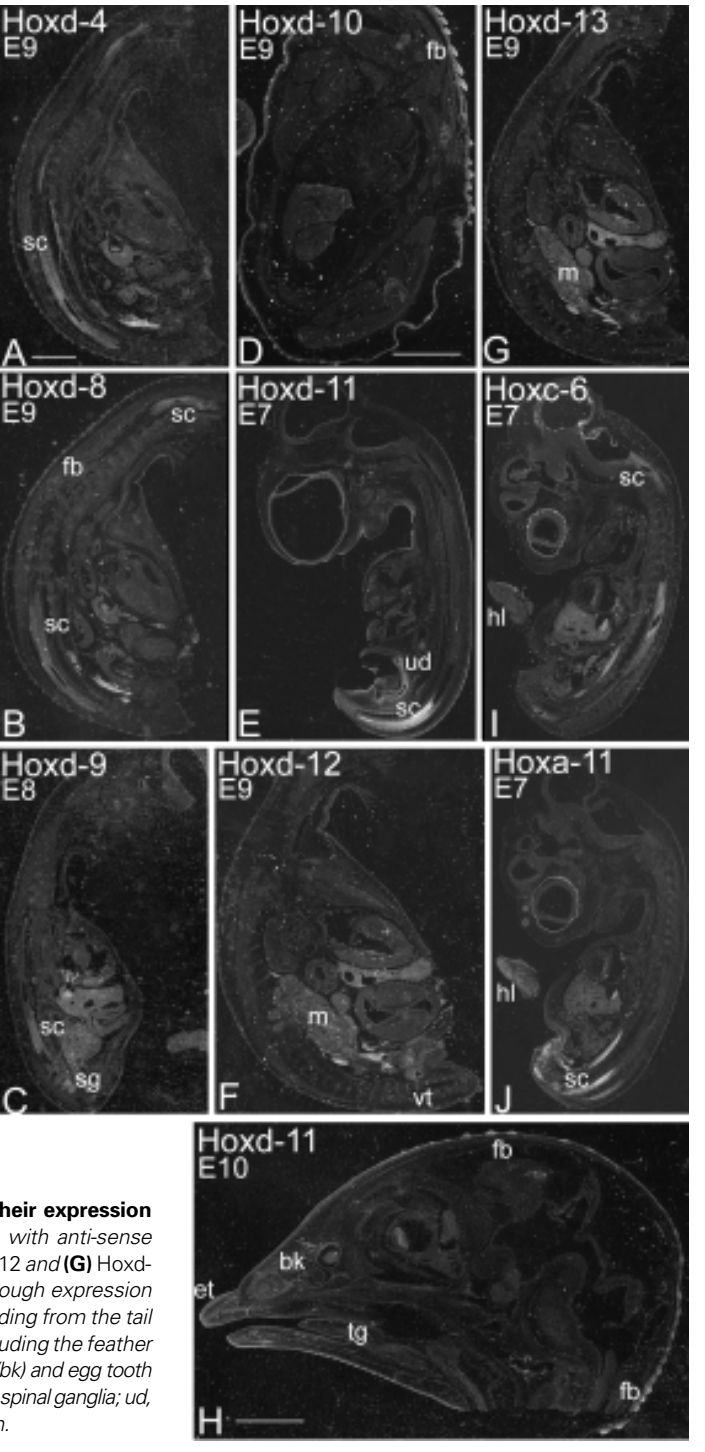
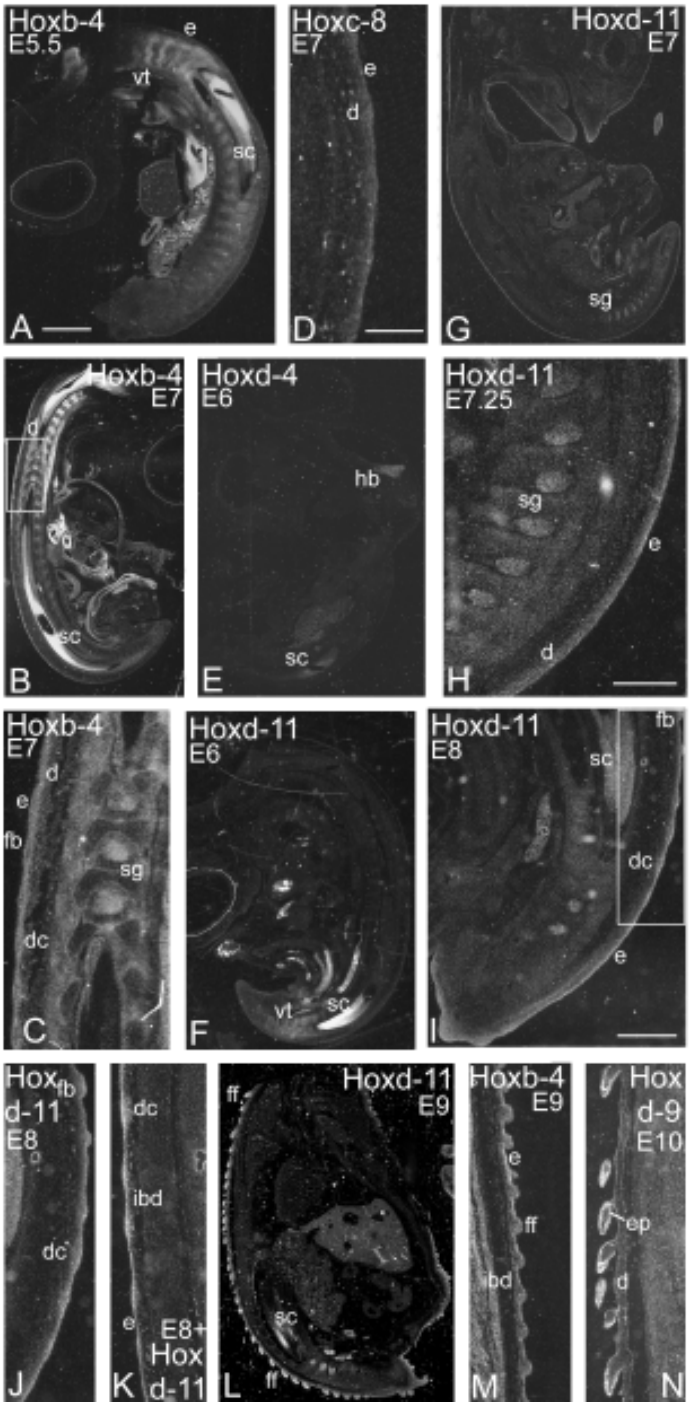


Fig. 3. *Hoxd* genes and some paralogues are regionally unrestricted in their expression within the developing chick skin. In situ *hybridization on sagittal sections with anti-sense probes.* **(A)** Hoxd-4; **(B)** Hoxd-8; **(C)** Hoxd-9; **(D)** Hoxd-10; **(E)** Hoxd-11; **(F)** Hoxd-12 *and* **(G)** Hoxd-13 *transcripts are detectable within the spinal cord (sc) and vertebrae (vt) although expression becomes sequentially posteriorized. In the skin expression is ubiquitous, extending from the tail region anteriorly to the head (A-G). Within the head* **(H)** *the entire skin region including the feather buds (fb) express* Hoxd-11*. Transcripts are also detectable in the tongue (tg), beak (bk) and egg tooth (et).* Hoxc-6 **(I)** *and* Hoxa-11 **(J)** *also exhibit ubiquitous expression within the skin. sg, spinal ganglia; ud, uteric duct; m, mesonephros; hl, hindlimb. Darkfield illumination. Scale bar, 2.0 mm.*



appears to be dynamic as initial expression is observed in an unspecific manner throughout the dermal territory (Fig. 4H). However, over the next 24 hours expression intensifies at the sites of dermal condensation immediately beneath the protruding feather buds (Figs. 4I and 4J). During this period transcripts become down-regulated within the inter-bud territories. At approximately E8 there is clear restriction of gene expression within the feather bud itself whilst adjacent regions of inter-bud dermis have little or no labeling (Fig. 4K). Apparently identical results were obtained for all the *Hoxd* genes examined (data not shown), and also for *Hoxc-6* and *Hoxa-11* (Figs. 3I, 3J, and not shown). All of these genes therefore commence their expression at the same time within epidermis and then, later, in dermis. We considered this surprising, as it is usually believed that the dermis holds the architectural blueprint and induces the epidermis to commit to a specific phenotypic fate.

Although there was a difference in both the initial timing and extent of expression of the two classes of *Hox* genes considered above (i.e., spatially restricted and spatially unrestricted), there were similarities in the patterns of expression within the expressing regions. Thus, as described above, expression commences in epidermis and then extends to include adjacent dermis. Expression was seen in both feather buds and feather filaments (See Fig. 4L for *Hoxd-11* and 4M for *Hoxb-4*). At later stages of feather development, gene transcription continues to be dynamic as expression intensifies within the epithelial sheath that overlies the mesenchymal cells of each developing feather, whereas intensity decreases within the inter-dermal zones (Fig. 4N).

Discussion

Spatial and Temporal Patterns of Hox *Gene Expression in Skin*

Earlier published work has indicated two distinct patterns of *Hox* gene expression in the skin: regionally restricted; found for chick and mouse *Hoxc-8* (Kanzler *et al.*, 1994, 1997) and mouse *Hoxd-9*, *Hoxd-11* and *Hoxd-13* (Kanzler *et al.*, 1994), and regionally unrestricted; found for mouse *Hoxc-13* (Godwin and Capecchi, 1998). Our present study on developing chick skin extends the above findings. Here we present evidence that confirms the data presented for *Hoxc-8* by Kanzler *et al.* (1997) but also extends this group to include *Hoxb-4* and *Hoxa-7*. All these genes are expressed in a structurally colinear fashion both in midline axial structures such as neural tube and vertebrae, and also within the skin. In the skin, these genes exhibit anterior boundaries of

Fig. 4. Temporal regulation of *Hox* genes during the development of the chick skin. In situ *hybridization of sagittal sections with anti-sense probes. (A,B,C and M)* Hoxb-4; *(D)* Hoxc-8; *(E)* Hoxd-4; *(F-L)* Hoxd-11 *and (N)* Hoxd-9. Hoxb-4 *transcripts are already detectable in the skin up to the level of cervical prevertebra 4/5 at embryonic day 5* **(A)**. *At this stage they are confined to the epidermis (e). By E7* **(B,C)**, Hoxb-4 *transcripts are also seen in the underlying dermis (d), especially within the dermal condensations (dc) underlying each feather bud (fb)* **(C)** *(box in B represents C). At E7, transcripts of* Hoxc-8 *can be seen both within the epidermis and the dermis* **(D)**. *At about E6 transcripts of* Hoxd-4 **(E)** *and* Hoxd-11 **(F)** *are present in the hindbrain (hb)* (Hoxd-4 *only), the spinal cord (sc) and vertebrae (vt), but not within the overlying skin. At E7* **(G)** *expression of* Hoxd-11 *maintains its anterior boundary within the spinal ganglia (sg) but is expressed ubiquitously throughout the overlying skin. At E7.25* **(H)** *transcripts of Hoxd-11 are clearly detectable within both the dermis and the overlying epidermis. Over the following 24 hours* **(I,J and K)** *expression is maintained in the epidermis but becomes dynamic within the dermis as expression becomes down-regulated within the inter-bud dermal territories (ibd) and up-regulated within the dermal condensations underlying each individual feather bud (box in I represents J). At E9,* Hoxd-11 **(L)** and Hoxb-4 **(M)** *transcripts are detected within individual feather buds and elongating feather filaments (ff). There is also a decrease of expression within inter-appendage dermal territories. However, the epidermis maintains transcriptional activity. At E10* **(N)** *the dermis is void of* Hoxd-9 *transcriptional activity. Expression is weakly maintained within the inter-appendage epidermis whilst being specifically intensified within the epithelial compartment of each individual feather filament. Darkfield illumination. Scale bars: A,B,E,F,G and L, 2.0 mm; C and I, 0.5 mm; D,H,J,K,M and N, 0.25 mm.*

expression that become progressively more posterior as chromosomal position becomes more 5' in location along their respective clusters. Thus the anterior limit of *Hoxb-4* expression is more anterior (pre-vertebra 4-5) than that of *Hoxa-7* (pre-vertebra 9), and this in turn is more anterior than that of *Hoxc-8* (pre-vertebra 19). In contrast, a larger group of genes (*Hoxd-4*, *Hoxd-8*, *Hoxd-9*, *Hoxd-10*, *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *Hoxa-11* and *Hoxc-6*) exhibit unexpected expression patterns within the chick skin. Structural colinearity (Duboule and Dolle, 1989) would predict an increasingly posterior restriction of expression as chromosomal position extends 3' to 5'. However, our results for genes of the *Hoxd* cluster show that each gene does not exhibit spatial restriction, but rather is expressed ubiquitously throughout the dorsal skin.

Temporally, there is further dichotomy in the regulation of *Hox* gene expression within the skin in that there seems to be two distinct profiles of activation. Here we present the first evidence that *Hox* genes regionally unrestricted in the skin do not follow the rule of temporal colinearity since all genes, *Hoxd-4* to *Hoxd-13*, commence expression concomitantly at E6.25. Our data are insufficient to demonstrate temporal colinearity amongst the genes that are spatially restricted in skin (*Hoxb-4*, *Hoxa-7* and *Hoxc-8*). We did find, however, that all genes of this group are already expressed by embryonic day 5.

It has often been assumed that the phenomena of structural and temporal colinearity are obligate features of the way in which *Hox* genes become activated, and that this is somehow linked to the way in which the expression patterns are generated (Duboule, 1992; Duboule, 1994). For example, a sequential opening of Hox clusters, 3' to 5', could account for the sequential activation and expression domains of the genes (van der Hoeven *et al.*, 1996; Kondo *et al.*, 1998). The fact that many *Hox* genes are expressed in skin without obeying structural or temporal colinearity shows that the clusters need not necessarily be activated in this way, and raises the question as to whether or not they are indeed so activated during gastrulation. In an alternative view of *Hox* activation, the clusters commence in an open state with all genes already accessible to transcription factors. This latter view would appear to be supported by the finding that *Hoxb-1* displays a rather normal pattern of expression even after its transposition to a 5' position within the *Hoxd* cluster (Kmita *et al.*, 2000).

The regionally unrestricted expression of *Hox* genes in skin can be considered as a late-onset expression (6.25 days in chick), with early-onset expression of *Hox* genes, necessary for establishment of the body axis, occurring much earlier (18 hours to 2 days in chick). Two important points are raised here. First, it seems likely that late-onset expression of *Hox* genes represents those situations where the genes have become co-opted for functions outwith their ancestral role (i.e., specification of the body axes). There is positive evidence for this in the case of *Hoxc-13*, whose expression is necessary for the normal development and strength of hair (Godwin and Capecchi, 1998). As noted by Duboule (1998b), it is likely that such co-option can occur only late in development, since early co-option would disturb normal axial development (Duboule, 1998b). Other examples of late-onset co-option of *Hox* genes might be in blood (van Oostveen *et al.*, 1999) and spermatogenic cells (Wolgemuth *et al.*, 1987; Lindsey and Wilkinson, 1996). A second important point is that our results question a common assumption that cell lineages late in development are refractory to the novel activation of their *Hox* genes as a consequence of prior blocking by the Polycomb repressor mechanism. This mechanism is generally assumed to maintain patterns of *Hox* expression in all cellular lineages after their first establishment at gastrulation (Yu *et al.*, 1998; Akasaka *et al.*, 2001). Clearly this assumption is not entirely valid, and some cell lineages, such as those in skin, can either wipe clean their Polycomb repression, or else are never subject to this repression in the first place.

The results we present here are generally based on a chick model system. However, interestingly, there seem to be differences in *Hoxd* gene expression patterns in the mouse. Kanzler *et al.* (1994) looked at the expression of *Hoxd-9*, *Hoxd-11* and *Hoxd-13* during murine skin morphogenesis. Here they found that these three genes were restricted in their expression to the epidermal cells of the most caudal skin regions. For mouse skin, we also confirmed the observation that *Hoxd-9* is regionally restricted and confined to epidermis and have observed that *Hoxd-12* is expressed in the same manner. This seems to contradict the findings we detail in this paper for the chick and sheds an interesting light on the potential differences between skin morphogenesis in the mouse compared to chick.

Commencement of Hox *Gene Expression within the Epidermal Layer*

Our results also cast an interesting light on the dynamics of expression within the two different layers of the chick skin: the epidermis and the dermis. Initial expression, whether it be restricted or unrestricted, is exclusive to the epidermis. Within the epidermis the initiation of expression of the restricted genes occurs by E5, whereas transcripts of the unrestricted genes are not detectable until E6.25. Strikingly, however, the expression within the dermis for both sets of genes commences at about E6.5, and expression here exactly mimics the expression within the epidermis. We considered that this latency of dermal expression could be due to the synchronized arrival of the dermal progenitor cells at the dorsal skin region at embryonic day 6.5. However, the dermis of the back has already formed by embryonic day 5 through the migration of somatic dermatomal cells (Mauger, 1972; Kanzler *et al.*, 1997).

Our observations may shed some light on the signaling interactions leading to the generation of morphogenetic cell fate. Classical heterogeneous recombination experiments have shown that the architectural blueprints of skin appendage morphogenesis are generally stored within the mesenchyme. For example, when feather mesenchyme is recombined with scale epithelial placodes the epithelium acquires a feather morphogenetic fate (Widelitz *et al.*, 2000 and references therein). It is clear from these experiments that the mesenchymal cells induce the competent epithelial cells to commit to a specific phenotype. Despite this, it seems plausible that early in skin development it may be that the epidermis signals to the dermis to initiate a developmental event. In this case, the epidermis, which already expresses *Hox* genes, may signal to the dermis to initiate *Hox* gene expression by means of a self or Hox-Hox regulatory mechanism (Hooiveld *et al.*, 1999). Evidence for epidermal to dermal signaling has already been shown. Kishimoto *et al.* (2000) showed that both Sonic hedgehog and members of the Wnt family, which are ectodermally restricted in their expression, signal from the epidermis to the dermis (Kishimoto *et al.*, 2000).

The Role of Hox *Genes in Developing Skin*

It is a likely possibility that *Hox* genes regionally restricted in their expression may play a role in the developmental patterning of skin and hair type. As examples, *Hoxd-13* apparently plays a role in the specification of chick footpads (Kanzler *et al.*, 1997), and mouse

Hoxc-8 may encourage hair growth specifically in the region of the posterior dorsal flank (Kanzler *et al*., 1994). However, knockout of *Hoxd-9* (Fromental-Ramain *et al*., 1996), *Hoxd-11* (Davis and Capecchi, 1994; Favier *et al*., 1995) and *Hoxd-12* (Davis and Capecchi, 1996) has not caused observable defects in the skin of mice. We note that the anterior boundary of *Hoxc-8* expression in chick skin, at the level of somite 22, corresponds to the transition between two different morphological compartments of the spinal pteryla (Sengel, 1976). Thus it is possible that *Hoxc-8* demarcates this specific anatomical division of the spinal pteryla.

The function of *Hox* genes that are regionally unrestricted in skin is more puzzling. In the mouse, we know that the *Hoxc-13* protein plays a role in the construction of normal hair. It seems unlikely to us that all of the genes that we have described as unrestricted in expression similarly play a role in feather morphogenesis. One possibility is that the genes play a role in patterning within individual developing feather buds. Chuong *et al*. (1990) has shown that the patterns of *Hoxc-6* and *Hoxd-9* expression are polarized in the feather follicle (Chuong *et al*., 1990). This suggests that different distributions of *Hox* genes within the follicle may pattern its spatial organization, rather like their roles in patterning the body and limb axes. However, our analysis of the regionally unrestricted gene expression patterns have not provided clear evidence that differences exist between them in their distribution of expression within the developing feather buds. An alternative possibility is that most of the regionally unrestricted *Hox* genes play no role in the development of the skin. Perhaps a few *Hox* genes, such as *Hoxc-13*, do have a role to play, but then their neighboring *Hox* genes simply become co-expressed without any purpose as a result of enhancer sharing (Gould *et al*., 1997; Mann, 1997) or by Hox protein activation of neighboring genes (Hooiveld *et al*., 1999). Such co-expression, without any overall purpose, might be a common feature of late-expressing *Hox* genes. This might, for example, explain why all, or most, of the *Hoxb* genes are co-expressed in developing lymphocytes (Quaranta *et al*., 1996).

Materials and Methods

Hoxd *Cosmids, Probe Preparation and* In Situ *Hybridization*

The *Hoxd* containing genomic clones (cos 4/6 and cos 2/2) were isolated from a chick cosmid library (Stratagene). Restriction enzyme maps were prepared by using a cosmid mapping kit (Amersham/Pharmacia). The *Hoxd* DNA templates used for probe preparation were as follows: *Hoxd-4*, described by Gaunt and Strachan (1994); *Hoxd-8*, a ca. 250 base probe (*Sma*1/*Apa*1 fragment) encompassing the homeobox; *Hoxd-9*, a 330 base probe (*Sau*3A/*Ava*1 fragment) extending 3' of nucleotide 15 in the homeobox; *Hoxd-10*, a 410 base probe extending 3' of nucleotide 99 in the homeobox; *Hoxd-11*, a ca. 500 base probe (*Pst*1 fragment) extending 3' of nucleotide 94 in the homeobox; *Hoxd-12*, a 330 base probe extending 3' of nucleotide 28 in the homeobox; *Hoxd-13*, a 410 base probe extending 3' of nucleotide 2 in the homeobox. Probes for chick *Hox* genes from other clusters were: *Hoxa-7*, described by Gaunt (2000); *Hoxa-11*, a ca. 500 base probe extending 3' of nucleotide 55 in the homeobox; *Hoxb-4*, described by Gaunt and Strachan (1996); *Hoxc-6*, described by Gaunt (1994); *Hoxc-8*, either complete coding region (Kanzler *et al*., 1997), or else a 255 base probe (*Sma*1/*Apa*1 fragment) encompassing the homeobox. *In situ* hybridization, using ^{35}S-labelled probes, was carried out as described earlier (Gaunt, 1987). Following *in situ* hybridization, sections were coated in Ilford (L4) photographic emulsion (gel form) and exposed at 4°C for 20 days. Slides were then developed, counterstained with Giemsa stain (BDH), mounted in DePeX (BDH) and analyzed using a Leica M420 microscope. Images were digitally photographed using a Leica DC 200 camera linked to a Leica MZ7$_5$ microscope. Each image was captured using Leica DC Viewer software. Images were then transferred to Adobe Photoshop.

Embryos

Fertilized chick eggs were supplied by Winter Egg Farm, Thriplow, Cambridgeshire. Embryos were washed in PBS, fixed in Bouin's fixative for between 2.5 and 5.5 hours and dehydrated in a graded series of ethanol. Embryos were then cleared in xylene and embedded in paraffin wax. 10 μm sections were then cut and floated on polylysine treated slides.

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