Gradients of homeoproteins in developing feather buds

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Summary

Homeoproteins are functionally involved in pattern formation. Recently, homeoproteins have been shown to be distributed in a graded fashion in developing limb buds. Here we examine the expression of homeoproteins in chicken feather development by immunocytochemical localization. We find that XlHbox 1 antigen is present in cell nuclei and is distributed in a gradient in the mesoderm of developing feather buds, with strongest expression in the anterior-proximal region. The gradient is most obvious in feather buds from the mid-trunk level. Feather buds from the scapular level express very high levels of XlHbox 1 and feather buds from the caudal region express no XlHbox 1, suggesting that a broad gradient along the body axis is superimposed on a smaller gradient within each individual feather bud. Feather ectoderm also expresses XlHbox 1 antigen but without an obvious graded pattern. Another homeoprotein, Hox 5.2, is also expressed in developing feather buds in a graded way, and its distribution pattern is partially complementary to that of XlHbox 1. These observations suggest that homeoproteins may be involved in setting up the anteroposterior polarity of cell fields at different levels, first for the body axis, then for the limb axis and finally for the feather axis.

Key words: homeoprotein, feather, pattern formation.

Introduction

Pattern formation is the process by which specific structures of the correct shape, size, position and orientation are generated. It has long been suggested that morphogenetic 'fields' and 'gradients' contribute to the establishment of patterns (Huxley and De Beer, 1934; Weiss, 1939; Wolpert, 1969; Malacinski and Bryant, 1984), but their molecular basis remains unknown. In Drosophila, homeobox genes are involved in pattern specification and are usually expressed in defined bands along the anteroposterior (A-P) axis (reviewed by Lewis, 1978; Gehring, 1987). Using low stringency hybridization, homeobox genes have been isolated from vertebrates (Carrasco et al. 1984) and shown to have position-specific expression patterns along the body axis (reviewed by Holland and Hogan, 1988; De Robertis et al. 1990). Recently, XlHbox 1 protein was shown to be distributed in a gradient in developing limb buds with strongest expression in the anterior and proximal region of the forelimb bud (Oliver et al. 1988b). Another homeobox gene product, called Hox 5.2, is also expressed as a gradient of nuclear protein in developing limb buds, but with the opposite polarity to that of XlHbox 1 (Oliver et al. 1989). In addition, there is orderly and sequential expression of genes of the Hox-5 complex along the distal to proximal axis of the limb, which correlates precisely with the 5' to 3' order of these genes in the genome (Dolle et al. 1989). Therefore, the same family of genes that controls the A-P body axis appears to also regulate limb axis formation.

Feather development is an excellent model to study the inductive processes that lead to the generation of complex structures because of their distinct patterns, accessibility to experimentation (reviewed by Lucus and Stettenheim, 1972; Sengel, 1976) and availability of mutants (Goetinck and Abbott, 1963). Feather buds express clear anteroposterior polarity and, although mesenchymal cells within a feather bud appear to be similar, there are molecular heterogeneities which are position-specific. For example, N-CAM is concentrated in the anterior part of the bud mesoderm (Chuong and Edelman, 1985a) while fibronectin is enriched in the posterior part of the bud mesoderm (Mauger et al. 1982). Because of the recent findings suggesting that homeoproteins are involved in determining patterns in vertebrates in several instances (Ruiz i Altaba and Melton, 1989; Kessel et al. 1990; Wright et al. 1989), we asked whether homeoproteins are also involved in feather pattern formation. First, are there homeoprotein gradients in feather buds? If so, are these gradients
reflecting the position the feather occupies along the body axis or the orientation of the feather axis?

In this study, we examined the distribution of homeoprotein X1Hbox 1 during feather development and found that there is indeed a graded distribution of X1Hbox 1 protein within individual developing feather buds. We compared the distribution of X1Hbox 1 with that of Hox 5.2 antigen and found a partially complementary pattern. The polarized distribution of N-CAM in feather buds was compared to the X1Hbox 1 gradient, and a partially overlapping, but clearly distinct, expression pattern was observed.

Materials and methods

Antibodies

Rabbit antibodies to X1Hbox 1 (or against its human homologue Hox 3.3.) and Hox 5.2 were made against fusion proteins and affinity purified as described in Oliver et al. 1988a and 1989. Rabbit antibodies against chicken N-CAM were prepared as described in Chuong and Edelman, 1985a.

Immunostaining

Immunostaining was processed as described in Oliver et al. 1988a. Briefly, chicken embryos were staged according to Hamburger and Hamilton (1951) and skin from different locations was dissected. Specimens were fixed in Bouin’s fixative, embedded in paraffin and sectioned at 6 µm. After de-paraffination, sections were incubated overnight with primary antibodies, followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega). NBT and BCIP (Promega) were used as substrate, with a positive reaction resulting in purple color.

Although endogenous alkaline phosphatase activity exists in the dermal condensation and feather buds (Hamilton and Konig, 1956) and we did observe it in our frozen sections of embryonic chicken skin, the paraffin embedding procedure we used completely abolished the endogenous alkaline phosphatase activity. This is most obviously shown in Fig. 3F, in which no dermal reactivity is seen after long incubation (1h) with substrate.

Chicken embryos

Fertilized eggs were obtained from Red Wing Farm (Los Angeles) and kept in an egg incubator (Humidare). We initially used White Leghorn chicken eggs in these studies. Later, we also examined a different strain (Sex-linked) of chicken embryos, and obtained the same results. Thus the expression is not specific to a particular strain.

Results

Normal feather development

The process of feather formation in the chicken embryo is briefly summarized in Fig. 1. For detailed descriptions, please refer to Lucus and Stettenheim (1972), Sengel (1976), Sawyer and Fallon (1983), and Mayerson and Fallon (1985). Feathers start as a flat sheet of ectoderm (Fig. 1A). The underlying mesoderm, called the feather field (according to the concepts of Weiss, 1939), acquires inductive properties and interacts with the ectoderm, initiating the differentiation of the epithelial feather placode (Fig. 1B). The placode epithelium undergoes rapid cell proliferation, while the mesenchymal cells beneath it increase in cell recruitment, as well as by cell proliferation (Chevallier, 1977; Gallin et al. 1986). Thus the growing feather bud has ectodermal and mesodermal components (Fig. 1C). The whole bud then invaginates into the skin to form the feather follicle (Fig. 1D) with the mesodermal core forming the dermal papilla and the feather pulp, and the bud epithelium becoming the collar and the feather filament. The collar is the induced epithelium where new epithelial cells are added to the growing feather (Fig. 1E). Therefore, in the feather filament, the tip is more mature than the base. The epithelium sheet invaginates and fuses, forming the alternating marginal plates and barb plates (Fig. 1F). The fused marginal plate cells later die, generating the space between barb plates. Most of the barb plate epithelium becomes keratinized and forms the feather barbs themselves, generating the branched pattern of the mature feather (Fig. 1G).
In adult feathers, the barbs (secondary branches) grow outward from the rachis (the primary branch, Fig. 1G), which is located in the anterior end of the feather bud. This side of the bud forms an obtuse angle to the body surface, while the posterior end forms an acute angle (Fig. 1C). It is important to note this because the anterior of the feather bud, the anterior of the body, and the anterior of the limb can point to different directions as development advances.

A homeoprotein gradient in the feather buds, which varies along the body axis

To examine homeoprotein expression in feather buds on the trunk along the anteroposterior axis, we prepared mid-sagittal sections of chicken trunks from stage 31 to stage 36 (embryonic days 7 to 10), when feather buds develop on the trunk. The sections were stained with anti-XIHbox 1 and an interesting pattern was observed (Figs 2, 3). At stage 31, XIHbox 1 expression in the mesenchymal cells of the forming dermis was graded along the anterior-posterior (A-P) axis of the body, with a sharp anterior border of intense staining and gradually decreasing amounts in more posterior regions (Figs 2B, 3A–C). This is reminiscent of the graded mesodermal pattern of XIHbox 1 protein observed in zebrafish embryos (Molven et al. 1990). At stage 34, the feather buds have formed. Feather buds from anterior parts of the trunk expressed high amounts of XIHbox 1 antigen. This expression gradually decreased toward the mid-trunk level and eventually disappeared in the buds from the caudal part of the body (Figs 2C, 3D–F). The most interesting observations came from mid-trunk region buds. Each of these expressed XIHbox 1 as a gradient, with higher amounts toward the anterior and proximal region of the bud (Fig. 3E). Mesodermic nuclei of feather buds from the scapular level expressed XIHbox 1 antigen so strongly that it is difficult to distinguish whether its distribution is graded or not (Fig. 3D). The pattern remained the same when we used excess amount of antibody to XIHbox 1, suggesting that the pattern is not due to insufficient amount of antibody. The unstained regions in the central cores of more mature feather buds (Fig. 3D,G) are newly formed blood vessels. Feather buds from the caudal regions expressed no XIHbox 1 in the mesoderm (Fig. 3F).

This variation in XIHbox 1 protein content in feather buds along the A-P axis of the body is not due to differences in the timing of A-P maturation of trunk feathers, which is known to proceed in an orderly way.

Fig. 2. Low power view of XIHbox 1 homeoprotein gradient in developing feather buds along the body axis. (A) Schematic drawing showing the orientation of the body (A, anterior; P, posterior; D, distal; V, ventral) and the corresponding positions from where higher magnifications were derived. Squares a (scapular level), b (mid-trunk level), c (caudal region), correspond to panels A,B,C of Fig. 3. Sagittal sections of stage 31, 34, 36 chicken embryo are shown in B, C, D. Note that XIHbox 1 expression is graded along the anterior–posterior axis of the body with the highest levels in the anterior body (B). As feather buds develop, the buds from the scapular region express XIHbox 1 throughout the mesoderm. Several buds in a row from the mid-trunk region express an XIHbox 1 gradient with higher levels in the anterior and proximal region of the feather buds (C,D, arrows). Tail feather buds even at this late stage do not express XIHbox 1 (D). Triangular, regions of artifacts. Bar, 1 mm. ×6.25.
Fig. 3. High-power view of XIHbox 1 homeoprotein gradient in developing feather buds along the body axis. Sagittal sections of stage 31 (A,B,C), 34 (D,E,F) and 36 (G,H,I) embryos. They are from scapular level (A,D,G), mid-trunk level (B,E,H), and caudal level (C,F,I) which correspond to squares a, b and c of Fig. 1A. In the feather placode stage, XIHbox 1 appears evenly distributed under the placode whether they are from scapular or mid-trunk region, although higher amounts are observed in those from the scapular region (A,B). The mesenchyme in the caudal region is devoid of XIHbox 1 (C). In later stages, while feather bud mesoderm from the scapular level expresses XIHbox 1 intensely (D,G). The core of feather bud is not stained because it corresponds to the region of newly formed blood vessels and will become feather pulp later.), the feather bud mesoderm from the mid-trunk level displays a gradient of XIHbox 1 maximal at the antero-proximal ends of feather buds (E,H, curved arrows). Tail feather bud mesoderm does not express XIHbox 1 (F) even at later stages (I), showing that the differences observed are not due to differences in feather maturation. The ectoderm expresses XIHbox 1 antigen in all regions. Some uneven distribution in epidermis was observed, but the precise pattern awaits further study. In both epidermis and mesenchyme, XIHbox 1 protein has a cell nucleus staining pattern. 100 μm. ×50.

(Mayerson and Fallon, 1985). This is known because very similar results were obtained when embryos of older stages were examined (Figs 2D, 3G–I). At stage 36, mesoderm nuclei from feather buds at the scapular level still expressed high amounts of XIHbox 1. The core region of the buds was negative because it was occupied by blood vessels (Fig. 3G). The graded pattern of XIHbox 1 within feather buds can be seen in several consecutive buds in Fig. 3H. Caudal feather buds, although well developed, were still entirely devoid of XIHbox 1 mesodermic staining (Fig. 3I) even at these late stages. The epithelial layer overlying each feather bud was positive for XIHbox 1 over the entire trunk region (Figs 2, 3). In some cases ectodermal expression seemed to be stronger in the posterior portion of the bud epidermis, but the pattern was not clear-cut and must await further studies. In both epidermis and
mesenchyme, the subcellular staining pattern of XIHbox 1 was nuclear. This is perhaps best seen in Fig. 4A for epidermis and Fig. 4B for mesenchyme. The nuclear localization raises interesting questions concerning the nature of the intercellular communication signals that establish a gradient of nuclear protein along an apparently homogeneous expanse of mesodermic cells.

We also examined the expression of XIHbox 1 in feather buds of the wing and observed an anterior and proximal gradient from the middle region of the wing. Feather buds from proximal regions of the wing expressed a large amount of XIHbox 1 in the mesoderm, while those from more distal regions contained much less XIHbox 1 protein (data not shown). Thus, the expression pattern of XIHbox 1 in the distal to proximal axis of the wing is analogous to the pattern of feather buds along the A-P axis of the body.

**Generation of the XIHbox 1 gradient in feather buds**

In this section we are solely concerned with development of feather buds from the dorsal feather area (Mayerson and Fallon, 1985) at the mid-trunk level, which normally display a clear XIHbox 1 gradient. The first indication of feather formation is the appearance of XIHbox 1 antigen in discontinuous dermal condensations (Fig. 4A left) in the feather field. As far as we can tell, these mesodermal changes precede any morphological changes in the ectoderm. The XIHbox 1 antigen is uniformly distributed throughout the feather field, even as the ectodermal placode begins to form. When the placode is more developed, XIHbox 1 begins to show higher expression in the anterior half of the feather field (Fig. 4A, right). When a distinct feather bud forms, the graded expression in the anterior and proximal mesoderm becomes obvious (Fig. 4B, curved arrows). Thus, apparently, the gradient is generated by decreasing the amount of antigen in the posterior mesoderm.

**Distribution of Hox 5.2 antigen in feather buds**

To explore whether other homeoproteins also express a similar graded distribution, we examined the expression of Hox 5.2 (Oliver et al. 1989). Similarly to the situation in the limb bud (Oliver et al. 1989), Hox 5.2 is negative in the ectoderm, but an interesting pattern is observed in the mesoderm (Fig. 5). In developing feather bud...
mesoderm, Hox 5.2 antigen is expressed very strongly throughout the feather field (Fig. 5A). Thus, all mesodermal cells in the feather field appear to express both homeodomain antigens at the early placode stage. As development proceeds, Hox 5.2 decreases in anterior and proximal regions of some feather buds (Fig. 5B, arrows), adopting a distribution that is opposite to that of XIIBox 1 (Fig. 5C). While this complementarity is clear in the feather buds shown here, in other buds it is only partial. This is due, at least in part, to the fact that Hox 5.2 is expressed throughout trunk as well as tail feathers, while the XIIBox 1 gradient is present only in feather buds of anterior and mid-trunk regions. Further studies are required to elucidate the complex patterns of Hox 5.2 in different feather areas. However, we can conclude that at least one other homeoprotein is expressed in the feather bud in a graded pattern, which in some buds is opposed to that of XIIBox 1.

**Comparative distribution of XIIBox 1 and N-CAM**

In our previous study, N-CAM was found to be expressed in a polarized way toward the anterior part of the feather bud (Chuong, 1985a). This posed an interesting question as to whether cells expressing XIIBox 1 in the anterior part of the bud coincide with those expressing N-CAM. We compared adjacent sections stained with anti-XIIBox 1 and anti-N-CAM (Fig. 6): N-CAM is expressed in the anterior portion of the bud mesoderm (Fig. 6A). In buds from the mid-trunk region, XIIBox 1 was expressed in some of the cells in the anterior bud mesoderm that expressed N-CAM, but the border of expression of XIIBox 1 extended more posteriorly than that of N-CAM (Fig. 6D, curved arrow). The staining seen in the posterior mesoderm of the buds here corresponds to the most anterior region of the next feather bud (Fig. 6A,D, inserts). In other sections, when the two buds were further separated, the enhanced expressions were seen to be located solely in the anterior buds (e.g. Fig. 4B). N-CAM was present on all the feather buds regardless of their position in the body. Feather buds from the caudal region still express N-CAM in the anterior part of the bud but were totally negative of XIIBox 1. This also served as a control, showing that the feather buds from the anterior part of the trunk are not entirely different from buds of the posterior part of
Fig. 6. Comparative distribution of XIHbox 1 and N-CAM during feather development. (A,B,C) N-CAM; (D,E,F) XIHbox 1. (A,D) Stage 34, sagittal sections; (B,E) stage 41, sagittal sections of feather follicles; (C,F) stage 41, cross sections of feather filaments. In the feather bud stage (A,D), N-CAM is present in the anterior (ant, straight arrow) feather mesoderm and part of the ectodermal placode as described previously (Chuong and Edelman, 1985a). The adjacent section shows that the distribution of XIHbox 1 overlaps partially with that of N-CAM but the two do not correlate exactly. The presence of XIHbox 1 extends to more posteriorly than that of N-CAM (curved arrow). N-CAM staining starts more anteriorly than that of XIHbox 1. Inserts are the lower power view which shows that the staining in posterior buds is due to the extension of the anterior end of the next buds. In others when two buds are wider apart, the enriched XIHbox 1 is seen in the anterior end only (Fig. 4B). In the feather follicle stage (B,E), N-CAM is concentrated in the dermal papilla (dp) and weakly expressed in the collar (cl). XIHbox 1 is enriched in the collar region but is also expressed in more distal ectoderm. Insert of E is the high magnification to show nucleus staining pattern of XIHbox 1 in collar region (square). In the feather filament (C), N-CAM is present in the marginal plate (mp) and absent in the barb plate (bp). An adjacent section (F) shows that XIHbox 1 has a complementary pattern: it is present in the barb plate and absent in the marginal plate. A,D, bar, 100 μm; ×125. B,C,E,F, and inserts in A and D, bar, 100 μm; ×50. Inserts in E, ×250.

As the feather bud continued to grow in height and the base of the feather bud begins to invaginate, forming the feather follicle, XIHbox 1 disappears from mesodermal cells but continues to be strongly expressed in nuclei of the collar region which is the ectodermal component of the proximal part of the feather where new epithelial cells are produced (Fig. 6E). The nuclear localization of XIHbox 1 in feather filament epidermis can be seen at higher magnifications (Fig. 6E insert). XIHbox 1 is negative in the mesenchymal components (dermal papilla and feather pulp) of the mature feather (Fig. 6E and F).

Interestingly, in the feather follicle stage, N-CAM and XIHbox 1 are expressed in a complementary way: N-CAM is concentrated in the dermal papilla (Chuong and Edelman, 1985b), the inducing mesenchymal component, while XIHbox 1 is absent from the dermal papilla but present in the collar epithelium (compare
Fig. 6B and E). In the proximal feather filament, N-CAM is present in the marginal plate cells (which are destined to die, forming the space between feather barbs) showing a dramatic spoke wheel pattern in transverse sections of the feather filament (Chuong and Edelman, 1985b, Fig. 6C). In contrast, XlHbox 1 is absent from the marginal plate cells but is present in the barb plate cells (Fig. 6F). Thus there is a complementary distribution pattern of N-CAM and XlHbox 1 in epidermis at later stages of feather development.

**Discussion**

In this study we provide evidence that the XlHbox 1 homeodomain protein is expressed as an anterior (and proximal) gradient during development of chicken feather buds of the mid-trunk region. This nuclear protein gradient is similar to that previously reported for XlHbox 1 in a very different structure: the forelimb bud mesoderm of Xenopus, mice and chicks (Oliver et al. 1988a, 1989). An anterior and proximal gradient is also present in the developing zebrafish pectoral fin bud (Molven et al. 1990). The conservation of homeobox proteins throughout vertebrate evolution enables one to use polyclonal antibodies to study homologous proteins in a variety of species, as discussed elsewhere (Oliver et al. 1989, Molven et al. 1990).

The intensity of XlHbox 1 staining in the individual feather buds varies with their position along the anteroposterior axis of the body. While feather buds in the mid-trunk region show the aforementioned gradient, those in the caudal region have no mesodermal staining, and feathers in the anterior trunk have XlHbox 1 antigen over most of the mesodermal region (Figs 2, 3). Thus, the amount of expression of this antigen in feather buds reflects its distribution along the body axis. Since feather formation follows a temporal sequence with anterior feather buds forming before more posterior ones in the dorsal feather area (Mayerson and Fallon, 1985), it was important to distinguish whether our observations were due to position-specific expression of XlHbox 1 or to different maturation stages of feather formation. Time course studies indicated that the mesoderm of feather buds from the posterior body do not express XlHbox 1 at any point of their differentiation (Fig. 3C,F,I). Thus the gradient of expression of XlHbox 1 protein in feather buds reflects a position-dependent property, not a maturation gradient that changes with time.

In the wing feathers, differences in expression intensity are also observed, with mesodermal expression being maximal in the proximal region and minimal at the distal tip. This is in agreement with the normal distribution of XlHbox 1 in the mesoderm along the proximodistal axis of the forelimb (Oliver et al. 1989). While the feather mesenchyme in the dorsal area of the trunk is thought to derive from somitic mesoderm (demonstrated by transplantation experiments using somites labelled with [3H]thymidine or transplanted from Japanese quail embryos, Mauger, 1972), the feather mesenchyme in the wing derives entirely from the somatopleure (Chevallier et al. 1977). Despite these different embryological origins, the XlHbox 1 gradient is present in feather buds in the trunk and in the wing.

It can be expected that other homeoproteins will also be expressed in feather buds. We have shown that a second homeobox antigen, Hox 5.2, is intensely expressed in feather buds, with a distribution clearly different from that of XlHbox 1. The distribution of Hox 5.2 antigen in some feather buds is complementary to that of XlHbox 1 (Fig. 5B,C). This is reminiscent of the complementary distribution of these two proteins during development of the forelimb bud (Oliver et al. 1989), in which XlHbox 1 protein is in the anterior proximal bud while Hox 5.2 adopts an opposite gradient with a maximum in the posterior and distal limb bud. The recent observation that several genes of the Hox 5 complex are coordinately expressed in the limb bud (Dolle et al. 1989) suggests that coordinated expression of other homeoproteins may also occur in developing feather buds. Presumably, in tail feather buds, which are totally negative for XlHbox 1, different homeoproteins may adopt the role performed by XlHbox 1 in the mid-trunk feather buds.

Our previous work had shown that the cell adhesion molecule N-CAM had a polarized expression in the feather buds, localized in particular to the anterior and proximal region (Chuong and Edelman, 1985a,b). The similarities of this distribution to that of XlHbox 1 in limb buds provided the initial reason for examining the expression of homeobox proteins during feather development. Careful examination of the pattern of N-CAM and XlHbox 1 expression (Fig. 6) has shown that, while the patterns partially overlap at the feather bud stage, they are distinct from each other. Furthermore, N-CAM has the same polarized distribution in feather buds from all regions of the body, while XlHbox 1 forms a gradient only in the mid-trunk region.

In the epithelial-mesenchymal interaction of feather formation, the pattern is determined by the mesenchymal components (Sengel, 1976). It would be interesting to explore what positional signals determine the extent of XlHbox 1 expression, how the homeoprotein gradient on the feather buds is set, and how this relates to the polarized expression of N-CAM. One candidate regulator, retinoic acid, is known to influence homeoprotein expression in teratoma cells (Mavilio et al. 1988). In some chicken strains, but not in all, retinoic acid can alter the morphology of scales, which become feather-scales (Dhouailly, 1984). This leads to an interesting question as to the causal relationship among retinoic acid, expression of homeoproteins, expression of N-CAM and the determination of feather patterns. The accessibility of feather development (Gallin et al. 1986; Goetinck and Carlone, 1988) to experimental manipulation makes it an ideal model for further work on pattern formation.

The first event in feather development we detected is the appearance of a group of ‘field’ of mesodermic cells that stains uniformly with homeobox antibodies. This precedes the appearance of an ectodermal placode. The
Homeobox gradient in feather buds


References

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(Accepted 14 September 1990)