

## 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 *XIHbox 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 *XIHbox 1* and feather buds from the caudal region express no *XIHbox 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 *XIHbox 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 *XIHbox 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, *XIHbox 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 *XIHbox 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 Lucas 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

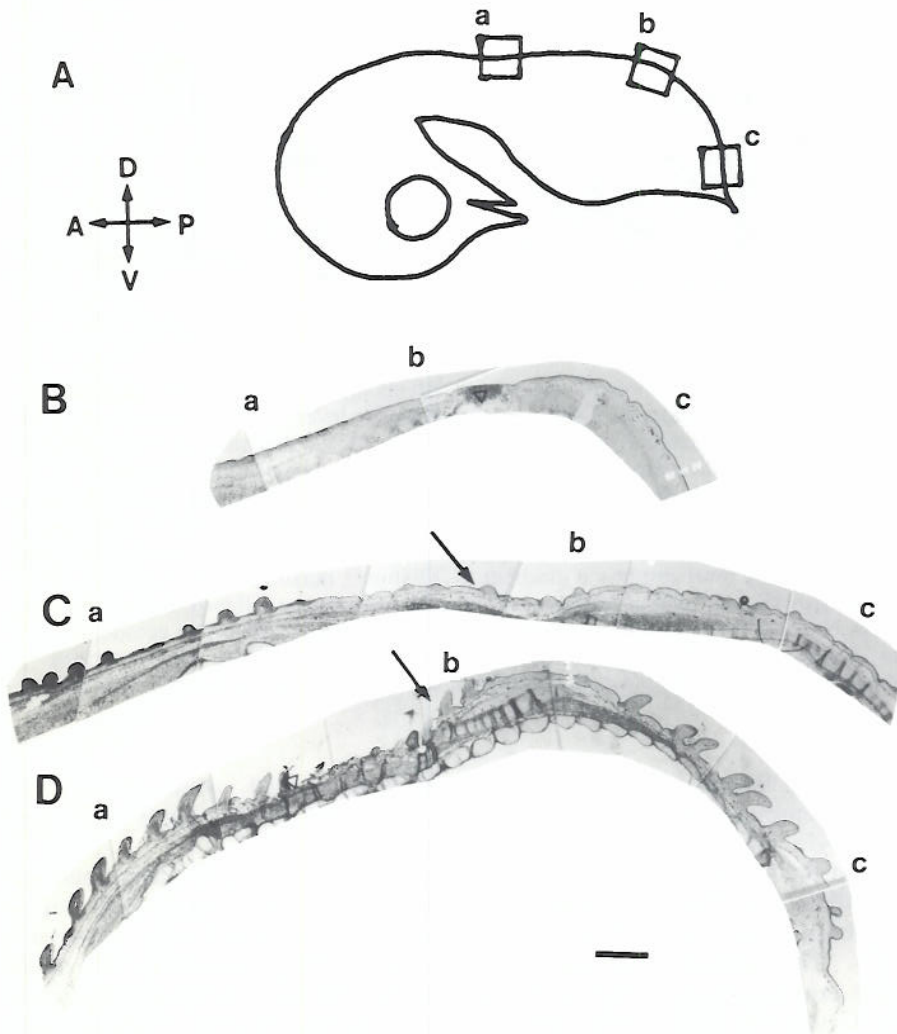
In adult feathers, the barbs (secondary branches) grow outward from the rachis (the primary branch, Fig. 1G), which is located in the anterior end of the feather bud. This side of the bud forms an obtuse angle to the body surface, while the posterior end forms an acute angle (Fig. 1C). It is important 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*

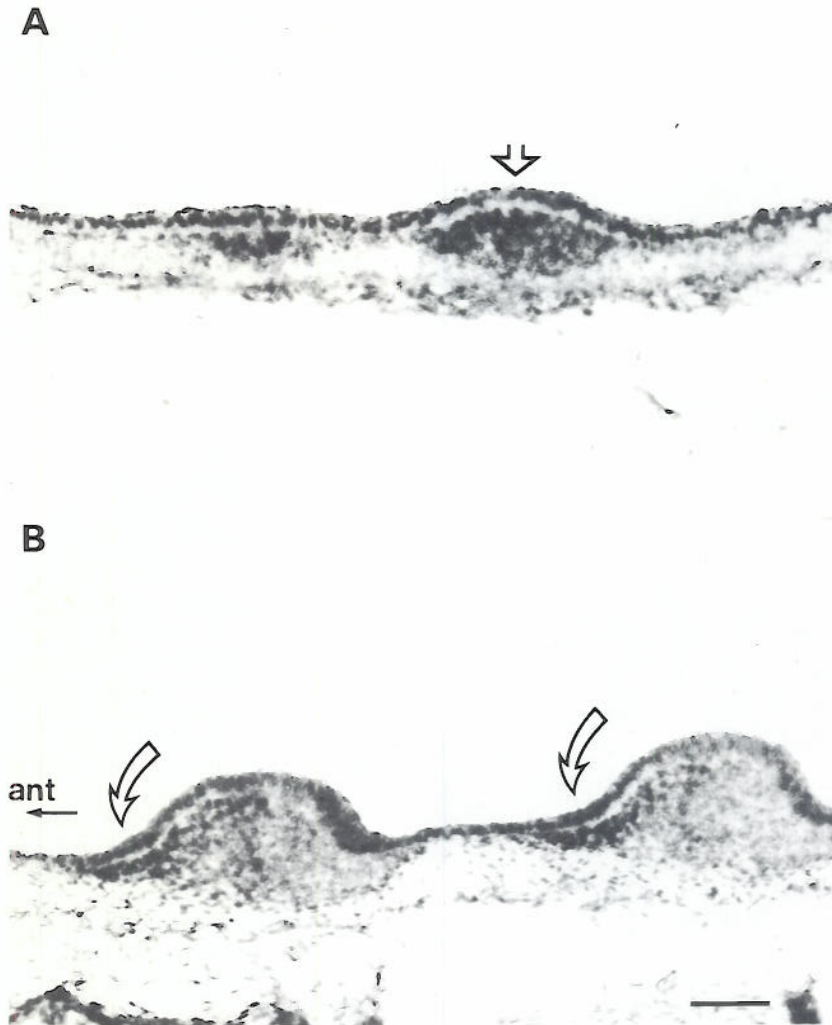
To examine homeoprotein expression in feather buds on the trunk along the anteroposterior axis, we prepared mid-sagittal sections of chicken trunks from stage 31 to stage 36 (embryonic days 7 to 10), when feather buds develop on the trunk. The sections were stained with anti-XIHbox 1 and an interesting 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.  $\times 6.25$ .



**Fig. 4.** Generation of the XIHbox 1 gradient in feather buds from a stage 33 embryo. Both panels were from the same embryo. Sagittal sections. (A) XIHbox 1 is expressed in the dermal condensation before the placode morphology in ectoderm is obvious (left feather germ in A). XIHbox 1 appears to be homogeneously distributed in the whole feather field at this stage, but the distribution gradually becomes polarized towards the anterior end of the feather germ. At the feather placode stage shown in the right feather germ in A, XIHbox 1 is more enriched in the anterior half of the feather field (straight arrow) than the posterior half. (B) As the feather buds form, XIHbox 1 becomes localized to the anterior (ant, small arrow) and proximal end of each feather bud (curved arrows). XIHbox 1 is also present on the ectoderm. Staining is exclusively nuclear in both ectoderm and mesoderm. The affinity-purified rabbit antibody was made against XIHbox 1 peptides derived from a cDNA clone of human origin (Oliver *et al.* 1988a). Bar, 100  $\mu\text{m}$ .  $\times 125$ .

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.

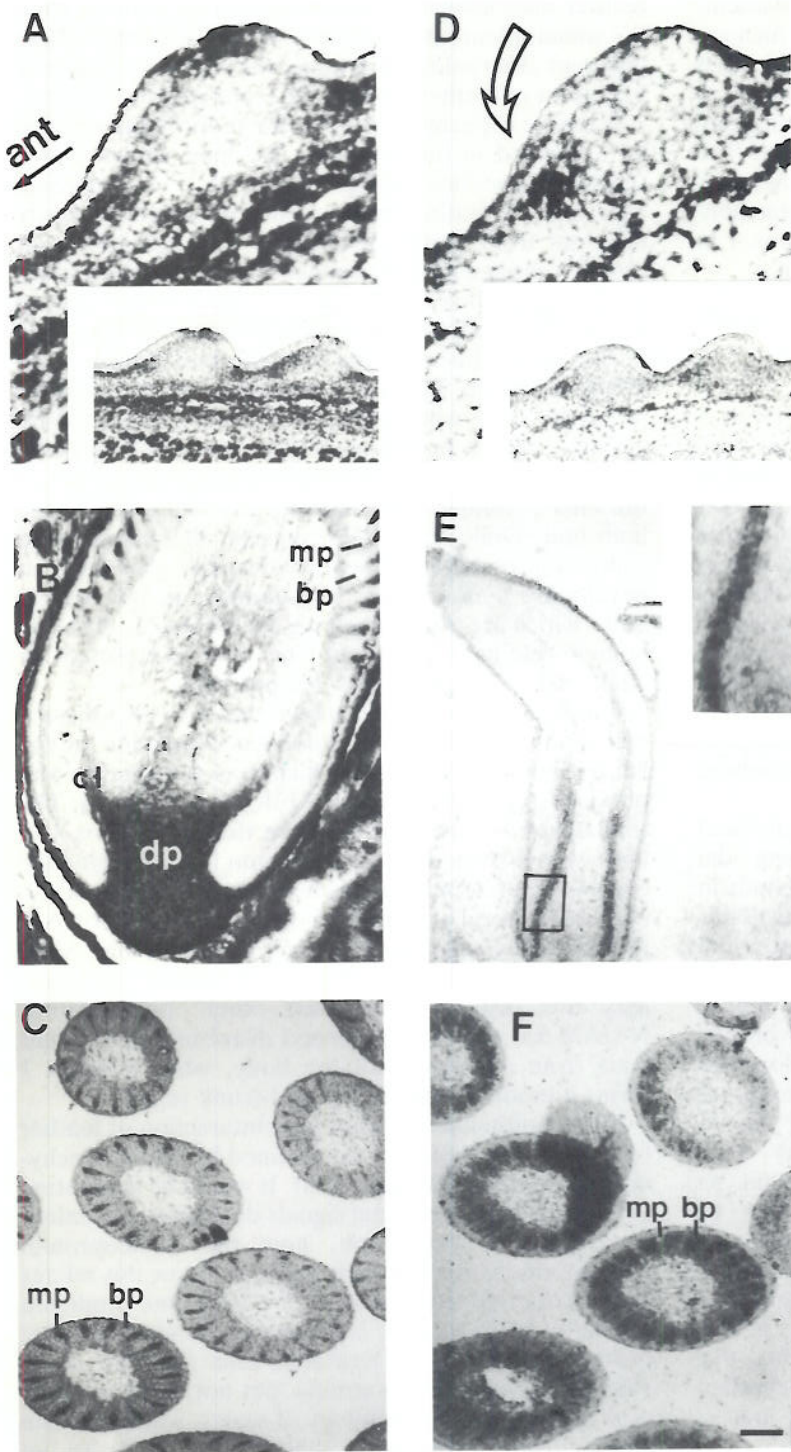
#### *Generation of the XIHbox 1 gradient in feather buds*

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



**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  $\mu$ m;  $\times$ 125. B,C,E,F, and inserts in A and D, bar, 100  $\mu$ m;  $\times$ 50. Inserts in E,  $\times$ 250.

the body, at least as far as the expression of a different molecule, N-CAM, is concerned.

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

homeodomain proteins (XIHbox 1 and Hox 5.2) only become graded after a feather bud has clearly formed (Figs 4 and 5). Based on the behavior of homeobox antigens during limb development, it has been proposed that homeobox genes may provide at least part of the molecular mechanism by which morphogenetic fields are established in embryos (Oliver *et al.* 1989). It is known (Harrison, 1918) that the mesodermal layer of the vertebrate embryo is divided at the neurula stage into 'morphogenetic fields' that give rise, after transplantation to a host embryo, to organs such as gills, forelimbs, hindlimbs and tails. It has also been proposed that each field would consist of a gradient of organogenic potential, or gradient-field (reviewed by Huxley and De Beer, 1934). XIHbox 1 is expressed as a band in the lateral plate mesoderm in amphibian embryos when the forelimb field is established (De Robertis *et al.* 1990). In the zebrafish, it then clearly forms a circular field of XIHbox 1 expression, which later acquires an anteroposterior gradient of protein, and finally becomes the pectoral fin bud (Molven *et al.* 1990). We show here that in later development, a new set of smaller fields appears during chicken feather formation and that each feather field is resolved into a mesodermal gradient of nuclear protein at the feather bud stage.

Thus, as has been discussed most clearly by Paul Weiss, in vertebrate development the embryo is subdivided into increasingly smaller fields of different developmental potential (Weiss, 1939). In the case of XIHbox1, the A-P body axis is first divided into a wide band of homeobox protein expression, which, in some cases, can be seen to gradually decrease towards the posterior end (Oliver *et al.* 1988b; Molven *et al.* 1990). Second, an anteroposterior gradient of XIHbox 1 expression is established during formation of the forelimb bud (Oliver *et al.* 1989). Third, a gradient of XIHbox 1 antigen forms along the anterior-posterior axis of the feather bud. The problem of pattern formation seems to be resolved into increasingly smaller fields, and homeodomain proteins may be utilized again and again in setting up the anteroposterior polarity of cell fields undergoing pattern formation.

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