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INVOLVEMENT OF THE *SONIC HEDGEHOG* GENE IN CHICK FEATHER FORMATION

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SUMMARY: To elucidate the molecular mechanisms of chick feather formation, we observed expression patterns of the *Sonic hedgehog* (*Shh*) gene, which is one of the vertebrate homologs of the *Drosophila* segment polarity gene, *hedgehog*, and encodes a signaling molecule functioning in limb pattern formation and motor neuron induction. We found that the *Shh* gene is also expressed in the apical region of the feather placodes and then in nine to eleven longitudinal stripes along feather filaments. The stripe was found to correspond to one of the outer marginal zones of each barb ridge, termed the zone of *Shh* expression. No significant expression signal was detected in the scale bud of developing legs. Thus, Shh is likely to function as an epithelial signaling molecule in epithelio-mesenchymal interaction during feather formation. Furthermore, since genes of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-4 (FGF-4) are coexpressed with *Shh* during feather formation as observed in limb morphogenesis, interactions among FGF-4, Shh and BMP-2 may be involved in formation of feather filaments and barbs in a similar fashion as elucidated in limb pattern formation.

Chicken skin consists of two major cutaneous structures, feathers and scales. In development of the cutaneous structures, epithelio-mesenchymal interaction is involved. It has been supposed that the ectoderm influences the placement of the mesenchyme, and then the mesenchyme condenses in particular regions and induces the overlying ectoderm to form the cutaneous structures (1). To understand the interaction between ectoderm and mesoderm at the molecular level, we have explored growth factors and their receptors involved in the epithelial-mesenchymal interaction during feather formation by performing *in situ* hybridization. We have found that genes of the FGF receptor type 1 and 2 are expressed differentially in feather buds, indicating that at least two members of the FGF family play differential roles in feather formation (2). Thus, although the mechanism for the epithelio-mesenchymal interaction is not fully elucidated yet, reciprocal interactions among various growth factors including members of the FGF family and the transforming growth factor-ß

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(TGF-ß) superfamily are likely involved in formation of the cutaneous structures (1, 2). Furthermore, a new factor of Sonic hedgehog (Shh) was recently found and demonstrated to interact with FGFs during limb pattern formation (3). Thus, in the present study, we examined whether the *Shh* gene is expressed during feather formation.

Shh is a vertebrate gene homologous to the *Drosophila* segment polarity gene, *hedgehog* (*hh*) (3). *Shh* has been reported to be involved in induction of the floor plate and motor neurons in the spinal cord, and in anterior-posterior pattern formation of the vertebrate limb (4 as a review). Since *hh* mediates cell to cell communication to establish specific patterns in *Drosophila* (5 as a review), Shh is supposed to be a crucial factor mediating intercellular interactions during vertebrate morphogenesis. In limb morphogenesis, Shh is supposed to induce expressions of *BMP-2* and *FGF-4* in limb buds (3). Thus, we searched feather buds for cells expressing *Shh*, *BMP-2* and *FGF-4* by means of whole-mount *in situ* hybridization. We found that *Shh* is expressed in feather placodes with concomitant expression of *BMP-2* and *FGF-4*, and then in the outer marginal zones of each barb ridge, but not expressed in scale-forming regions of developing legs. These results suggested that Shh is involved in pattern formation of chick feathers, probably cooperating with FGFs and BMPs.

MATERIALS AND METHODS

Embryos. Chick embryos of White-Leghorn were incubated at 38°C. Stages were determined by the limb shape after Hamburger and Hamilton (6).

Cloning of chick* Shh *cDNAs. A cDNA fragment of *Shh* was cloned by a PCR method with primers described by Riddle *et al.* (3). With the PCR-amplified fragment, a cDNA library from chick embryos at stage 20 was screened to obtain a cDNA clone containing the entire cording region. The cDNA was subcloned into pBluescript SK(+).

In situ *hybridization.* Digoxigenin-labeled riboprobes were prepared according to manufacturer's instructions, using the chick *Shh* cDNA as a template. Whole-mount *in situ* hybridization was performed as described by Wilkinson (7). Stained embryos were sectioned as described by Sasaki and Hogan (8). To obtain probes for transcripts of the chick BMP-2 and chick FGF-4, a fragment of each cDNA was cloned by a PCR method, using degenerate primers encoding the corresponding conserved amino acid sequences.

RESULTS

Expression patterns of* Shh *during feather filament formation. The process of feather formation in the chick embryo is briefly illustrated in Fig. 1 (1). To observe expression patterns of *Shh* in developing limb and feather, we performed whole-mount *in situ* hybridization. Figure 2 shows typical results. At stage 23, signals stained as dark blue were observed in the posterior margin of the limb bud, known as the zone of polarizing activity (ZPA) (Fig. 2A). The expression in the ZPA became weak at stage 26 (Fig. 2B). Since our results coincided with those as demonstrated by Riddle *et al.* (3), we concluded that we were able to detect expression of *Shh* with our probe. At stage 31, expression of *Shh* became intense in the tip of feather buds in the dorsal area. The order in which *Shh* begins to express in feather buds is identical to that in which feather buds begin to appear, *i.e.*, the order is as follows: 1) the dorsal region at stage 31, 2) the thigh, tail and breast region at stage 33, 3) the humoral, ventral and wing regions at stage 34 (9). At stage 36, *Shh* signals

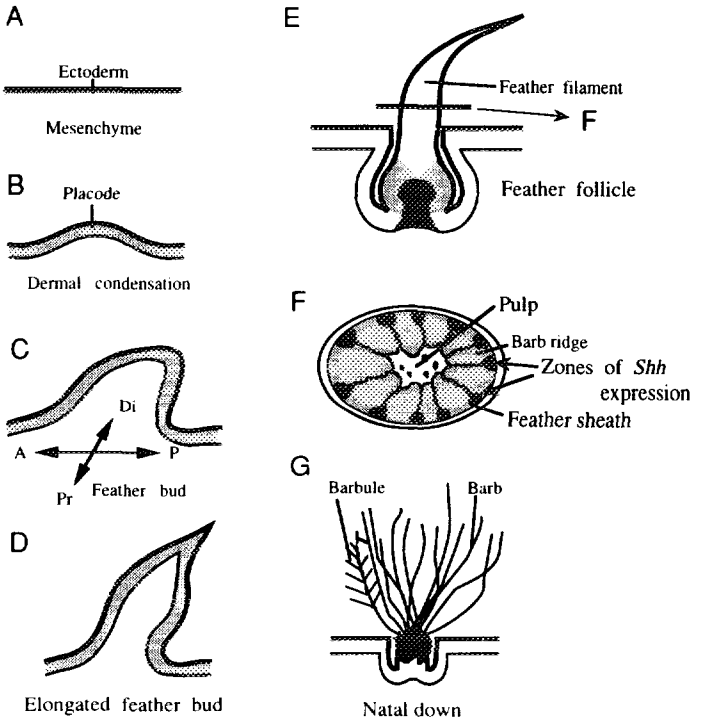

Fig. 1. Illustration of the feather formation process (1). **A**, Feather formation starts as a flat sheet of ectoderm; **B**, The underlying mesoderm interacts with the ectoderm, inducing the epithelial feather placode, while dermal condensation occurs in the mesoderm; **C** and **D**, The growing feather bud has both ectodermal and mesodermal components. A, anterior; P, posterior; Pr, proximal; Di, distal; **E**, The whole bud invaginates into the skin to form the feather follicle and the feather filament. A cross section of the feather filament is shown in F; **F**, The epithelial sheet invaginates and fuses, forming the barb ridges, while the mesoderm core forms the feather pulp. In the barbs, the secondary branched structures are induced to form barbules; **G**, Finally, natal downs are matured.

were detectable in the tip of wing feather buds (Figs. 2C, 2E and 2F), whereas no significant signal was detectable in the scale-forming region of the leg (Figs. 2G and 2H). At these stages, both *BMP-2* and *FGF-4* were expressed diffusely in the feather placodes (Figs. 2D and 3G, respectively). Sections of the stained feather buds reveal that the expression of *Shh* was restricted within the apical region of feather placodes (Fig. 2F), implying that *Shh* is presumed to be an epithelial factor involved in the epithelio-mesenchymal interaction between the feather placode and the condensed dermis.

Expression patterns of* Shh *in barb ridges. Figures 3A and 3B show expression patterns of feather filaments at stage 38. A sagittal section of a short filament reveals that expression of *Shh* is restricted to an epidermal layer in a distal half of the filament (Fig. 3C). A transverse section of a feather filament at the tip indicates that signals for the *Shh* expression were localized in the epidermal layer (Fig. 3D). As the epidermal layers differentiate into barb ridges in elongated feather filaments, the *Shh* gene is expressed in nine to eleven longitudinal stripes (Figs. 3A and 3B). The stripe was found to correspond to one of the outer marginal zones of each barb ridge (Figs. 3E and 3F), termed the zone of *Shh*

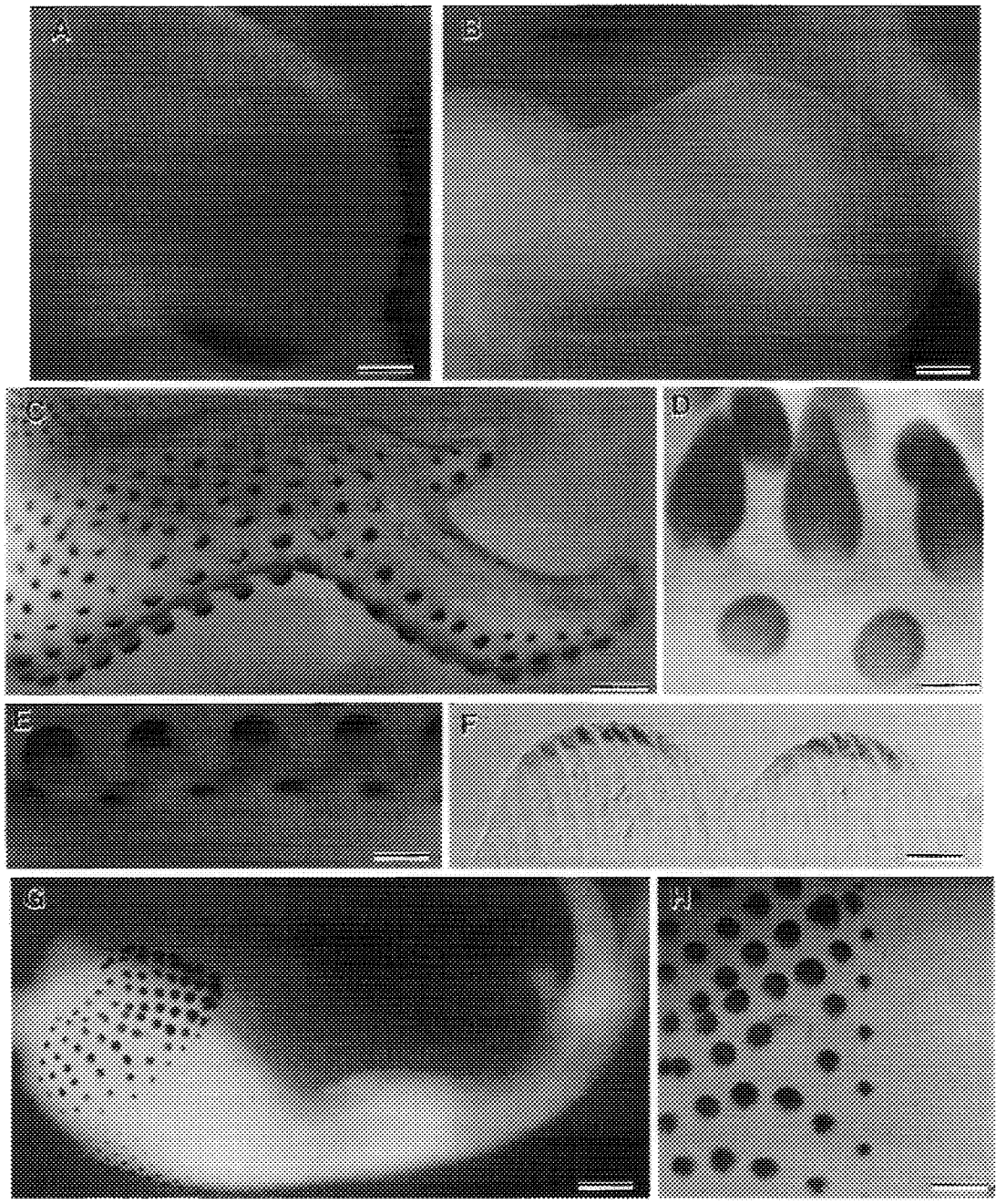

Fig. 2. Expression patterns of *Shh* during feather formation, as revealed by whole-mount *in situ* hybridization. A, a wing bud at stage 23; B, a wing bud at stage 25; C, a wing at stage 35; D, a wing at stage 35 with probe for *BMP-2*; E, an enlarged view of feather buds; F, a transverse section of feather buds; G, feather buds of a leg; H, an enlarged view of boundary between feather buds and the scale forming region (right). Bars: A, 200 μm; B, 300 μm; C, 520 μm; D, 150 μm; E, 200 μm; F, 1 mm; G, 1 mm; H, 300 μm.

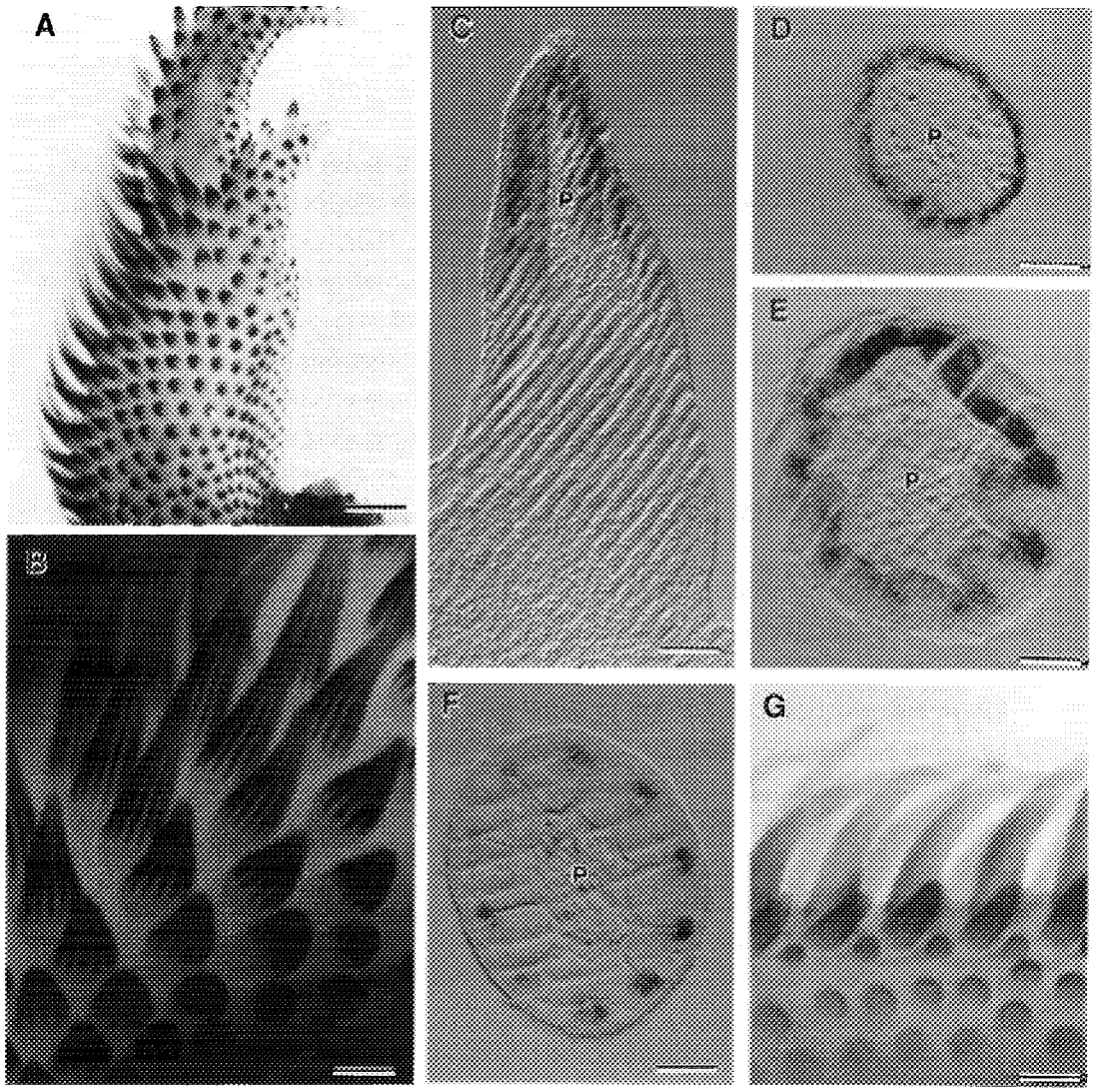

Fig. 3. Expression patterns of *Shh* during barb formation, as revealed by whole-mount *in situ* hybridization (ISH). A, a wing at stage 36; B, an enlarged view of *Shh* expression pattern at stage 37; C, a parasagittal section of an ISH-stained feather filament; D, a transverse section (tip) of an ISH-stained feather bud; E, a transverse section of an ISH-stained feather filament; F, a transverse section of an ISH-stained feather filament, indicating that *Shh* is expressed in the outer marginal zones of each barb ridge. The corresponding zone is termed the zone of *Shh* expression.; G, an expression pattern of *FGF-4* in wing feather at stage 36. p, feather pulp. Bar: A, 750 μm; B, 200 μm; C and D, 50 μm; E, 60 μm; F, 50 μm; G, 500 μm.

expression. No significant expression of *Shh* was observed at the base of the feather filament. The signals became progressively weak as the barb differentiated at later stages. No significant expression of *Shh* was observed, when barbules were formed in the barb. On the other hand, expressions of both *BMP-2* and *FGF-4* were observed diffusely in the epidermal layer of the feather filament (Figs. 2D and 3G, respectively). From these results, Shh is

likely to function as a signaling molecule involved in differentiation of barbs of the feather filament.

DISCUSSION

In the present study, we demonstrated that *Shh* is expressed during feather formation. It is intriguing to note that *Shh* is expressed repeatedly in the following order: 1) Hensen's node, 2) notochord, 3) spinal cord, 4) limb buds, 5) feather buds, and 6) barb ridges. In *Drosophila*, expression of *hh* is observed in early embryos as well as in the imaginal disks of wings and legs (5 as a review). The repeated expression of *hh* in the various pattern forming regions imply that the *hh* gene encodes an intercellular signaling molecule in pattern formations. As demonstrated by Riddle *et al.* (3), since *Shh* codes a crucial factor for pattern formation in the limb itself, Shh is likely to function as a signaling molecule involved in various patterning processes in vertebrates. In analysis of the epithelio-mesenchymal interaction during feather formation, the *Shh* expression should be an excellent marker for the feather placode.

It is well known that interaction between the apical ectodermal ridge (AER) and the ZPA is essential for the limb pattern formation (4 as a review). Epithelial factors from the AER are presumed to be FGF-4 (10), bFGF (11), FGF-8 (12), BMP-2 (13), and BMP-4 (13), whereas Shh and BMP-2 are likely to be mesenchymal factors from the ZPA. Thus, interactions among Shh, BMPs and FGFs should be indispensable for the limb pattern formation. Since *Shh*, *BMP-2* and *FGF-4* are also expressed in feather buds, interactions among the three may be essential for feather formation as elucidated in limb pattern formation.

Since feather formation is known to be induced in the scale epidermis when a scale-forming placode is recombined with the feather dermis, dermal factor(s) might induce the feather placode (1 as a review). It is also known that incubation of leg scale cells in the medium containing retinoic acid results in phenotypic change to feather (1 as a review). Furthermore, retinoic acid gradient was demonstrated to modulate the phenotype and orientation of a feather (14), suggesting one of the feather-inducing factors might be related with retinoic acid. Since retinoic acid is known to induce *Shh* expression in mesenchymal cells beneath the AER in the limb bud (3), the change of feather pattern by retinoic acid may be due to induction of *Shh* expression. In any case, Shh should be an intercellular signaling molecule in epithelio-mesenchymal interaction during feather formation.

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