Local Inhibitory Action of BMPs and Their Relationships with Activators in Feather Formation: Implications for Periodic Patterning

Han-Sung Jung,[*,1] **Philippa H. Francis-West,**[*,2] **Randall B. Widelitz,**[†]
Ting-Xin Jiang,[†] **Sheree Ting-Berreth,**[†] **Cheryll Tickle,**[*]
Lewis Wolpert,[*] **and Cheng-Ming Chuong**[†,3]

†*Department of Pathology, School of Medicine, University of Southern California, HMR 204,*
2011 Zonal Avenue, Los Angeles, California 90033; and *Department of Anatomy and*
Developmental Biology, The Medawar Building, University College London,
Gower Street, London, WC1E 6BT, United Kingdom

The formation of periodic patterns is fundamental in biology. Theoretical models describing these phenomena have been proposed for feather patterning; however, no molecular candidates have been identified. Here we show that the feather tract is initiated by a continuous stripe of *Shh, Fgf-4,* and *Ptc* expression in the epithelium, which then segregates into discrete feather primordia that are more strongly *Shh* and *Fgf-4* positive. The primordia also become *Bmp-2* and *Bmp-4* positive. Bead-mediated delivery of BMPs inhibits local feather formation in contrast with the activators, SHH and FGF-4, which induce feather formation. Both FGF-4 and SHH induce local expression of *Bmp-4,* while BMP-4 suppresses local expression of both. FGF-4 also induces *Shh.* Based on these findings, we propose a model that involves (1) homogeneously distributed global activators that define the field, (2) a position-dependent activator of competence that propagates across the field, and (3) local activators and inhibitors triggered in sites of individual primordia that act in a reaction–diffusion mechanism. A computer simulation model for feather pattern formation is also presented. © 1998 Academic Press

Key Words: feather; skin appendages; SHH; BMP; FGF; periodic pattern formation; reaction diffusion.

INTRODUCTION

A major question in embryonic development is how cells and tissues become precisely arranged to make up the body plan. One of the simplest and frequently observed patterns is the maintenance of a minimum distance between repetitive neighboring elements, namely periodic patterning (Wolpert, 1971; Wolpert and Stein, 1984). This is observed in teeth, hairs, feathers, digits, integument color patterns,

etc. Do these elements appear periodically from the beginning (prepatterned), or do they first appear homogeneously and then become periodically distributed through developmental progression? One of the major hypotheses on how periodic patterning can be generated is by the differential diffusion of chemical substances described in the Turing model (Turing, 1952). Turing showed that an initially homogeneous system of two or more diffusible chemical "morphogens" could develop periodic heterogeneity after small, random disturbances. The concept gave rise to the idea that diffusible signaling molecules in combination with random intrinsic instability may be enough to generate periodic patterns in a biological system. Meinhardt (Meinhardt, 1982; Koch and Meinhardt, 1994) further suggested that randomly generated initiation sites can produce both diffusible activators and inhibitors. With activators acting within a short range and inhibitors acting at a long range, it is possible to generate a stable periodic pattern. Oster and

[1] Current address: Developmental Biology Program, Institute of Biotechnology, University of Helsinki, Finland, FIN 00014.

[2] Current address: Department of Craniofacial Development, Guy's Medical and Dental School, 28th Floor, London Bridge, SE1 9RT, UK.

[3] To whom correspondence should be addressed. Fax: 213 342-3049. Web site: http://www-hsc.usc.edu/~cmchuong. E-mail: cmchuong@zygote.hsc.usc.edu.

Harris later expanded the hypothesis to suggest that it is also possible to generate periodic patterns by having mechanochemical forces behave in a Turing fashion, probably through cell motility and cell–cell/cell–matrix adhesion (Murray *et al.,* 1983; Oster *et al.,* 1983). Although Turing patterning has been demonstrated in a chemical model (Dulos *et al.,* 1996), no specific molecules and interactions have been worked out completely in a biological model.

Avian feather morphogenesis is a favored experimental model for pattern formation because alternating feather bud and interbud domains are arranged in a highly ordered array (Sengel, 1976; Sengel, 1990; Chuong, 1993). Many of the signaling molecules such as SHH,[4] FGFs, FGFR, BMPs, etc. are found to be expressed in feather buds (Nohno *et al.,* 1995; Chuong *et al.,* 1996). Among them, retroviral mediated ectopic expression of SHH has been shown to cause enlarged feather buds (Ting Berreth *et al.,* 1996a). Bead-mediated delivery of FGF-1, FGF-2, and FGF-4 has been shown to induce merged feather bud domains surrounding the bead and extra feather buds from the abdominal apteric region (Widelitz *et al.,* 1996). FGF-2 was also shown to induce feather buds from avian scaleless mutants (Song *et al.,* 1996). Since these molecules can increase the size and number of feather buds, they are considered activators for feather bud formation.

How does the interbud domain form? Is it simply a leftover from the bud domains? Is it induced by an interbud inducer that then becomes an inhibitor for feather bud formation? If inhibitors are acting to induce the interbud domains, are they produced directly in the interbud domain or are they produced in the bud domain and redistributed by diffusion? If the Turing hypothesis is functioning to establish the bud and interbud domains, the inhibitors would be expressed in the bud domain.

Among signaling molecule candidates, BMPs are the vertebrate homologues of Drosophila decapentaplegic (dpp) and have been found to be involved in the patterning of the limb bud, early Xenopus embryo, etc. In the limb bud, BMPs and FGFs have been shown to have antagonistic actions (Niswander and Martin, 1993), while FGFs are required for the expression of SHH (Laufer *et al.,* 1994). In Drosophila, dpp and hedgehog function in conjunction to elicit pattern formation (Zecca *et al.,* 1995; Mullor *et al.,* 1997). These results suggest that SHH, FGFs, and BMPs often work together in forming signaling loops in organogenesis. It is therefore pertinent to examine the roles of BMPs in feather formation and to study their relationship with SHH and FGFs.

In this study, we first use whole-mount *in situ* hybridization to examine the expression of these signaling molecules. We have particularly examined the early stages (before stage 30) of feather primordia formation which have not been reported before. We expose feather explant cultures to BMP-2 and BMP-4 locally released from beads to examine the

roles of these growth factors in feather bud formation. These results are compared with the results from studies with FGFs and SHH-coated beads. The ability of these signaling molecules to regulate the expression of each other is also examined. Finally, we present a model on feather periodic patterning, incorporating previous models and molecular candidates.

METHODS

In Situ Hybridization

Digoxigenin-labeled nucleotides were incorporated into RNAs transcribed *in vitro* from linearized cDNAs for use as riboprobes. *In situ* hybridization was carried out as described in Sasaki and Hogan (1993) and used in our laboratory for embryonic chicken skin (Ting-Berreth and Chuong, 1996a). Following hybridization, the tissues were incubated with anti-digoxigenin Fab conjugated to alkaline phosphatase (Boehringer-Mannheim). Positive *in situ* hybridization signals were detected by incubation with NBT/BCIP substrates (Promega) in alkaline buffer.

Immunocytochemistry

Immunostaining was done according to Chuong *et al.* (1990). Antibody to the N terminal of SHH is from Bumcroft *et al.* (1995).

Explant Cultures

Dorsal skins from stage 26–31 (Hamburger and Hamilton, 1951) White Leghorn chicken embryos (SPAFAS) were dissected in Hank's buffered saline solution (HBSS, Gibco/BRL) and transferred to culture inserts (Falcon) in six-well culture dishes (Falcon). Explants were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) containing 2% fetal bovine serum (Irvine Scientific). Growth media were placed in the outer well and a thin layer of media was placed within the culture insert to keep the tissue moist. The explants were grown at the air–media interface at 37°C in an incubator containing 100% humidity and an atmosphere of 95% air/5% CO_2. The developmental progression of the explants was monitored with an inverted microscope (Olympus IMT-2). Photographs were taken with an Olympus OM-4 camera.

Growth factors were added to local regions of the cultures by soaking beads in the growth factors and then placing them on top of the explants. For this procedure, about 100 Affi-gel blue beads (Bio-Rad, 100–250 μm in diameter) were washed with sterile phosphate-buffered saline and soaked in 5 μl of growth factor for 1 h at 37°C following the procedure of Hayamizu *et al.* (1991) and used in our laboratory for TGF-β (Ting-Berreth and Chuong, 1996b). The beads were carefully manipulated into place on the skin explants using fine forceps. Growth factor beads were stored for up to 1 week at 4°C. Control beads were soaked in the same concentration of bovine serum albumin.

RESULTS

Feather Bud Activators, Shh and Fgf, Are Initially Expressed as a Continuous Stripe in the Primary Row, While Bmps Are Later Expressed Directly in a Punctate Pattern

To study the initial events in feather formation, we focused on the very early stages of feather morphogenesis in

[4] Abbreviations used: SHH, Sonic hedgehog; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; Ptc, Patched.

the lumbosacral region of chicken skin using whole-mount *in situ* hybridization. Here we examined the expression of several genes at stage 28, before feather primordia became apparent (Sengel, 1978). Surprisingly, we observed a continuous stripe of *Shh* transcripts in the midline where the primary row of feather buds will form (Fig. 1A, arrow, midline is designated by the open arrow). The continuous linear pattern breaks into distinct units at stage 29 (Fig. 1B, arrowhead) and then propagates bilaterally by stage 33 (Fig. 1C). The staining sharpens and increases in the individual placodes. *Ptc* (*Patched*), a target of *Shh* signaling (Goodrich *et al.,* 1996; Marigo *et al.,* 1996), also appears as a linear pattern initially at stage 28 (Fig. 1D, arrow) and then becomes expressed periodically by stage 29 (Fig. 1E). Chicken skin is divided into many tracts, each with its own primary row. The linear pattern of *Shh* is also observed in other feather tracts, including the femoral and caudal tract (Fig. 1I), suggesting this is a general phenomenon.

While *Fgf-8* is absent in the skin (not shown), *Fgf-4* has been shown to be expressed in the feather buds (Nohno *et al.,* 1995; Widelitz *et al.,* 1996). At stage 28, we found that there is also a linear *Fgf-4* stripe that has not been reported before (Fig. 1F, arrow). In contrast, *Bmp-2* and *Bmp-4* are completely absent in the corresponding midline stripe at stage 28 (Fig. 1G). Later, *Bmp-2* and *Bmp-4* appear directly and periodically in each feather primordia (Figs. 1H and 1N). *Bmp-2* and *Bmp-4* are present in both the epithelium and mesenchyme, although the relative distribution seems to shift over time. In the early short feather bud, *Bmp-2* is expressed at higher levels in the mesenchyme, while *Bmp-4* is enriched in both epithelium and mesenchyme (Figs. 1O and 1P). When feather buds become asymmetric later, *Bmp-2* becomes enriched in the anterior mesenchyme (Fig. 1H, arrow).

How are the signaling molecules distributed in the primary row? A cross-section of the midline strip shows that *Fgf-4* and *Shh* are present in the epithelium at this stage (Figs. 1A–1C, arrowhead). *Ptc,* downstream to SHH, is mainly in the epithelium, but is also seen in the mesenchyme. *Bmp-2* and *Bmp-4* are negative (Fig. 1G and not shown). Later, when feather primordia and interbud space form in an alternating pattern, *Shh* becomes restricted and more strongly expressed in the distal placode epithelium (Nohno *et al.,* 1995; Ting-Berreth and Chuong, 1996a) and disappears from the interbud regions. This disappearance is consistent with our earlier finding that *Shh*-positive placodes, when separated from patterned mesenchyme, lose *Shh* and placode morphology in 3 h (Chuong *et al.,* 1996). *Fgf-4* also becomes restricted in the primordia region and starts to be expressed in the bud mesenchyme (Widelitz *et al.,* 1996). *Ptc* transcripts shift to the distal mesenchyme with lighter staining remaining in the epithelia (Fig. 1M).

SHH, FGF4, and BMPs are signaling molecules and it would be helpful to know the distribution of the protein. Here we show the distribution of SHH protein using antibodies against SHH (Fig. 2). In the placode stage SHH is present in the distal placode epithelium. In the short feather bud stage, SHH is expressed in the tip of the bud epithelia. In the long bud stage, SHH is in the distal end of the feather bud epithelia. These expression patterns are essentially the same as those for the transcripts detected *in situ* (compare with Fig. 1 of Ting-Berreth and Chuong, 1996). In the mesenchyme, there is some diffuse SHH immunoreactivity particularly in the later feather bud stages. This may represent the accumulated SHH proteins in the bud region.

In summary, the expression patterns show that in the very early stages of feather formation, the primary row starts as a continuous stripe that is positive for *Fgf-4, Shh*, and *Ptc.* This stripe then breaks into periodic feather primordia with increased *Fgf-4* and *Shh* expression in the primordia and disappearance in the interbud regions. At this time, the periodic primordia become positive for *Bmp-2* and *Bmp-4*.

FIG. 1. Expression of signaling molecules during the initial stages of feather development. (A–I) Whole-mount *in situ* hybridization. Size bar, 500 μm. (A) Top view showing linear expression of *Shh* transcripts in the epithelium along the midline (arrow) at stage 28. Open arrow pointing in the posterior direction, midline. (B) *Shh* expression is altered from a linear to a punctate pattern, appearing from posterior to anterior at stage 29. Anterior to the arrowhead, *Shh* transcripts remain linear. (C) At stage 33, *Shh* expression is localized within the epidermal placode of each feather bud. (D) Top view of *Ptc* expression in the back of a stage 28 embryo. *Ptc* also appears as a single stripe in the midline (arrow). Note posterior expression is more apparent than anterior. (E) Stage 29. *Ptc* became localized in each feather bud. (F) Top view of *Fgf-4* expression in the midline (arrow) on the back of a stage 28 embryo. The staining, although present, is not as strong as those of somites (arrowheads) positive for *Fgf-4*. (G) Stage 28. *Bmp-2* transcripts are not detected in the back, femoral, or tail regions of the embryo. (H) Stage 33. *Bmp-2* transcripts appear in the feather buds. A side view at this stage showed that it is confined to the anterior feather bud mesenchyme. A bud domain is marked by two small arrows. (I) Lateral view of a stage 29 embryo. *Shh* is expressed as a line in the femoral tract (arrow) and caudal tract (arrowhead) which will become the primary row of each tract. (J–L) *In situ* hybridization on transverse sections. Midline transverse sections. Size bar, 50 μm; except N, 200 μm. (J) Stage 29. *Fgf-4* is localized in the epithelium of the primary row in the midline. Note the placode-like morphology of the epithelium. There is no obvious mesenchymal condensation at this stage. The spinal cord is marked by the broken line. (K) Stage 30. There is a slight protrusion of the primary row. *Shh* expression is localized to the tip of the placode-like epithelium. The spinal cord (SC) is marked by the broken line. (L) Stage 28. *Ptc* appears strongly in the primary row epithelium (arrowhead) and weakly in the mesenchyme and other epithelia. The spinal cord is marked by the broken line. (M) At stage 31, *Ptc* transcripts shift down to the mesenchyme. Some weak staining remains in the placode. (N) Overview of *Bmp-4* in the spinal tract. Note that the more lateral region (toward lower panel) is completely negative, then *Bmp-4* appears directly in the feather primordia. (O) *Bmp-4* is localized in the feather primordia, more in the mesenchyme at stage 30. The dashed line delineates the epithelium. (P) *Bmp-2* is expressed in both epithelium and mesenchyme of the feather primordia at stage 30. The dashed line delineates the epithelium.
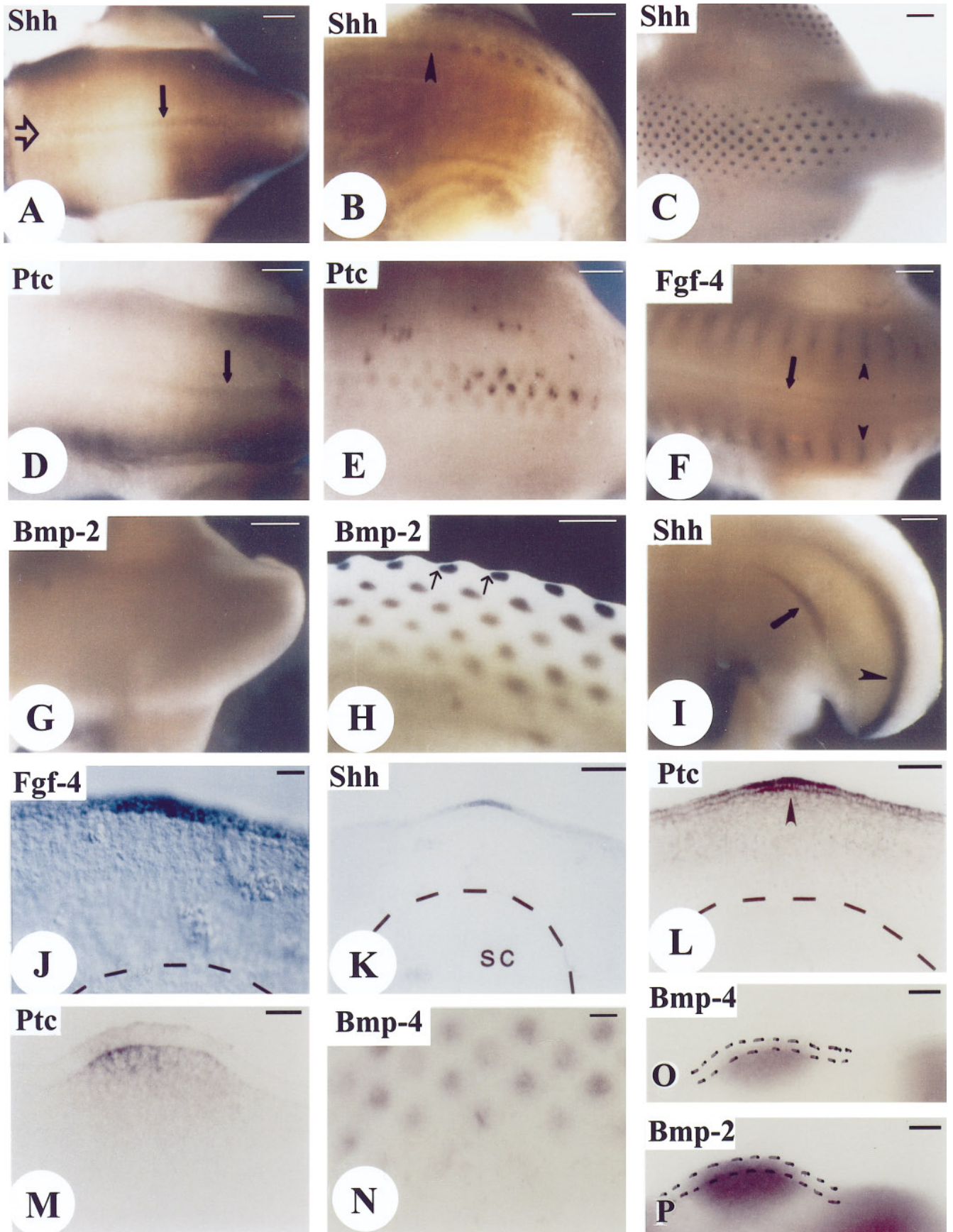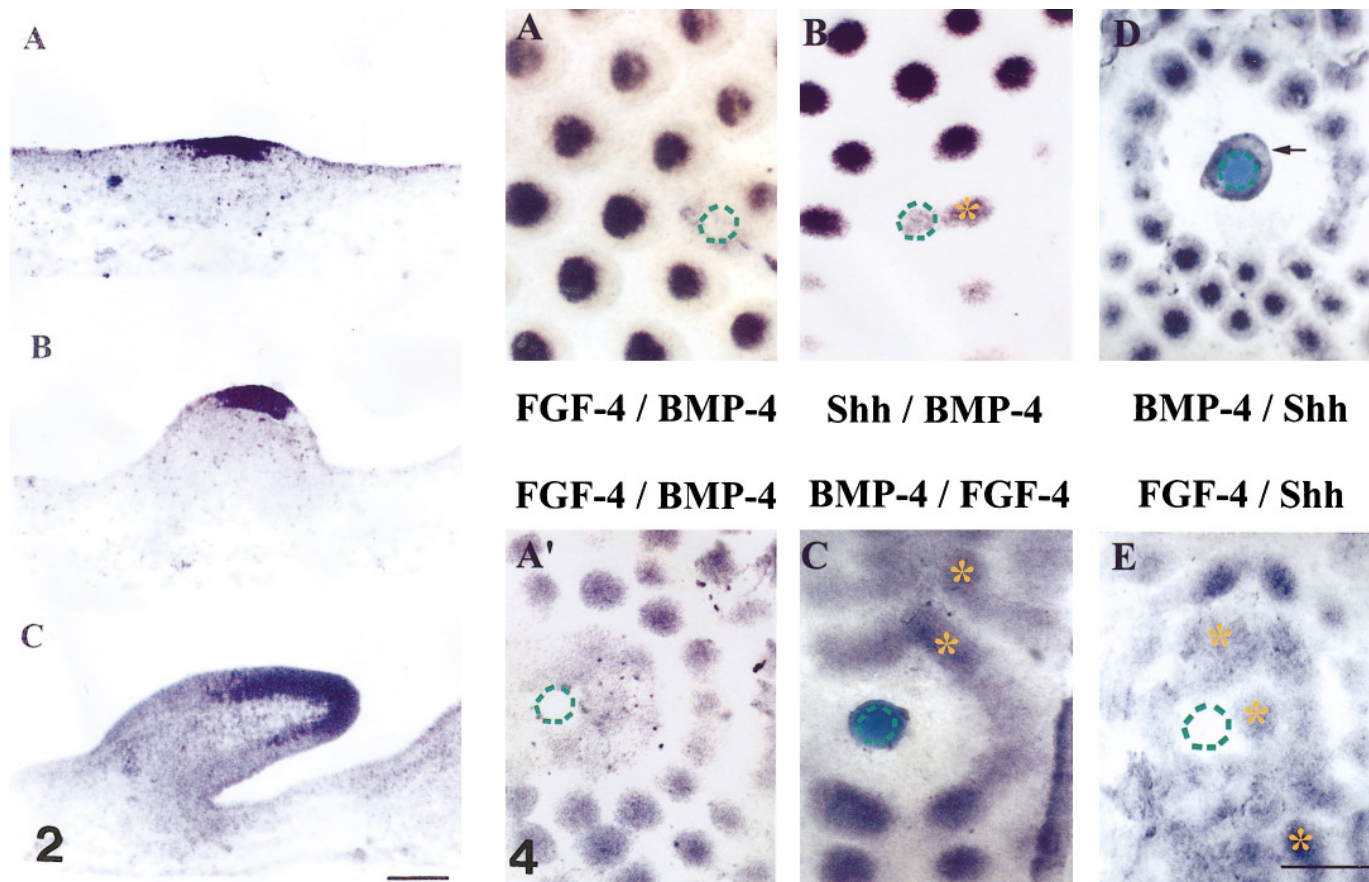
FIG. 2. Expression of Shh protein in developing feather buds. Paraffin sagittal sections of stage 31–36 chicken embryos through the developing buds of dorsal skin. Young feather buds at (A) stage 31, (B) stage 34, and (C) stage 36 expressed Shh protein. Shh protein is strongly positive in the distal epithelia, similar to the *in situ* hybridization pattern (Ting-Berreth and Chuong, 1996). Some faint and diffuse Shh immunoreactivity can also be detected within the buds particular at stage 36. Bar, 100 μm.

FIG. 4. Effect of SHH, FGF-4, and BMP-4 on each other. Note that the bead or where the bead was in each panel is indicated by a circle. In A, B, and E, the beads were dislodged during *in situ* hybridization preparation. Some previous buds near the bead are indicated by asterisks. (A, A′) FGF-4-soaked bead (850 μg/ml) was placed for (A) 6 or (A′) 16 h in the midline on the dorsal skin. *Bmp-4* transcript was detected around the bead. Broken line indicates the extent of the induced BMP-4. Scale bar, 400 μm. (B) SHH-soaked bead (1 mg/ ml) was placed for 6 h in the midline on the dorsal skin and *Bmp-4* transcript was detected at the position of the bead. The staining right to the bead is from a previous bud (*). Scale bar, 250 μm. (C) BMP-4-soaked bead (660 μg/ml) was placed for 16 h on the dorsal skin, which caused the inhibition of feather buds around it. *Fgf-4* transcript was downregulated around the bead. Scale bar, 400 μm. (D) BMP-4-soaked bead (660 μg/ml) was placed for 16 h on the dorsal skin, which caused the inhibition of feather buds around it. *Shh* transcript was downregulated around the bead, except the regions immediately adjacent to the bead (arrow). Scale bar, 400 μm. (E) FGF-4-soaked bead (850 μg/ml) was placed for 16 h in the midline on the dorsal skin. SHH transcript is induced in the tissue around the bead. The image appears heterogeneous because it is a mixture of induced SHH which is diffusive and the original SHH which is present in the previous feather buds. The tissue is also undergoing reorganization, and the buds will eventually merge as seen in Fig. 3E. Scale bar, 400 μm.

Delivering BMPs with Beads Suppresses Feather Bud Formation Locally

To study their effects locally, beads were soaked in BMP-2 and BMP-4 solution (1 μg/ml to 1 mg/ml), picked up, and placed on stage 29–32 skin explants. We observed the inhibition of feather bud formation around the bead (Table 1, Figs. 3 and 4). Use of the bead alone does not induce or suppress bud formation (e.g., see Ting Berreth and Chuong, 1996b). Beads soaked in 1 μg/ml BMPs have no detectable effects. Since the bead is 200 μm in diameter and the interbud zone is about 100–150 μm, there will be no detectable difference if the inhibitory zone is less than 400 μm. Beads soaked in 10 μg/ml start to show an inhibitory zone of about 400 μm. At and above 333 μg/ml, the zone of inhibition reaches approximately 400–800 μm in diameter. With

TABLE 1

Local Inhibitory Effect of BMPs on Feather Bud Formation

	Concentration[a]	n[b]	Percentage of response		
			<400	400–800	800–1200[c]
BMP-4	1	12	100	0	0
	10	10	60	40	0
	33	24	25	67	8
	333	13	0	62	38
	666	12	0	0	100
	1000	33	0	0	100
BMP-2	1	18	89	11	0
	10	16	57	43	0
	333	17	0	88	12
	666	10	0	75	25
	1000	20	0	40	60

[a] Beads were soaked in the indicated concentration of growth factor. This reflects the relative amount of BMPs delivered, not the absolute concentration of BMP delivered to the tissue, which is probably much less. See results for further discussion.

[b] Number of beads. This is compiled from several independent experiments.

[c] Diameter of the inhibitory zone in micrometers. Since the bead is 200 μm and the interbud space is about 100–150 μm, a zone below 400 μm is considered as no detectable inhibition.

666 μg/ml, the size of the zone of inhibition is in the range of 800–1200 μm in diameter, and higher concentrations do not cause further increases in size. This may also represent the saturation of the bead's capacity to carry BMPs.

For bead-mediated growth factor delivery, it has been difficult to ascertain exactly how much is released from the bead to the adjacent tissue. This is a complex phenomenon. Although we have immersed the bead in 1 μg–1 mg/ml BMP-4 solution (Table 1), the real amount being delivered to tissues is likely to be much lower since the growth factors must go through the processes of adsorption to the bead and balancing between protein–media, protein–bead, and protein–tissue interactions. We still describe the experimental conditions in terms of the concentration that was used to soak the bead, so that different laboratories can compare results with this procedure. Similar effects were observed for BMP-2 (Table 1).

Previously we showed that FGF-1, -2, and -4 proteins induce more feather buds from early skin or competent apteric regions and induce expanded bud domains from more mature skin (Widelitz *et al.,* 1996). Here we observed another phenomenon. In the early stages, when FGF-4 beads (0.85 μg/ml) were placed around the midline, a ring of feather bud domain was induced (Fig. 3C and Widelitz *et al.,* 1996). However, when FGF-4 beads were placed in the lateral regions of dorsal skin at stage 29, no response was observed (Fig. 3D). In contrast, lateral skin at stage 31 can respond to FGF-4 beads (Widelitz *et al.,* 1996). These results suggest that the response to FGF-4 depends on the position in the skin, which in turn may reflect a propagating maturation gradient of competence to respond to FGF-4. In contrast, the suppressive effects of BMP-4 and BMP-2 are the same in the midline regions and in lateral regions (Fig. 3B).

To examine histological changes produced by BMP-4 treatment, we prepared sections of the explant across the BMP-4 beads. We found that the epithelia adjacent to the BMP-4-coated bead became thickened due to the formation of multiple epithelial cell layers (Fig. 3F). This increase is not seen in control beads or in beads soaked in other growth factors (see for example, Ting-Berreth and Chuong, 1996b, Fig. 6E′). During normal skin development, the interfeather follicular epithelium becomes multilayer epidermis. Therefore, BMPs may prevent the formation of feather buds by inducing epidermis that is resistant to feather bud activators. There is no tissue necrosis adjacent to the BMP bead. Phosphorylated CREB (cAMP responsive element binding protein) has been shown to be expressed in some regions of the epithelium and feather bud mesenchyme, but not the interbud mesenchyme (Noveen *et al.,* 1995). The section was also stained with antibody to pCREB. Consistent with the finding here, the region surrounding the bead is negative for pCREB.

Previously, we have shown that ectopic RCAS-mediated *Shh* expression in skin induces large feather buds *in ovo* (Ting-Berreth and Chuong, 1996a). Here we further test the direct effect of SHH protein. When SHH-coated beads were placed near the midline of stage 29 skin explants, there was an enormous increase of the feather bud domain in a range of about 800 μm diameter around the bead (Fig. 3E).

Regulatory Relationship between Feather Bud Activators and Inhibitors

Do these activators and inhibitors regulate each other? To test for this possibility, we used *in situ* hybridization to examine the effect of different growth factors on the expression of each other. When a FGF-4 bead was placed near the midline of dorsal skin for 6 h, a small region of *Bmp-4* expression was induced, which reached a range of approximately 800 μm in diameter at 16 h (Figs. 4A and 4A′). When a SHH bead (1 mg/ml) was placed in the interbud region near the midline of dorsal skin and removed at 6 h, a localized expression of *Bmp-4* was already induced (Fig. 4B). If the SHH bead is left longer, a wider zone of *Bmp-4* can be observed (not shown). In contrast, a BMP-4 bead (1 mg/ml) inhibited the expression of both *Shh* and *Fgf-4* in a zone of about 1000 μm in diameter at 16 h (Figs. 4C and 4D). Immediately around the BMP bead, there is a thin rim of high *Shh* expression, which indicates that BMP-4 may induce *Shh* at a high concentration.

We did most of the *in situ* hybridization at 16 h when the tissue around the bead is undergoing tissue reorganization. Therefore, the expression pattern of signaling molecules tends to be heterogeneous. This is because it is composed of the induced expression that is diffusive and the periodic staining from the previous buds. The diffusive staining can

BMP4   **FGF4**
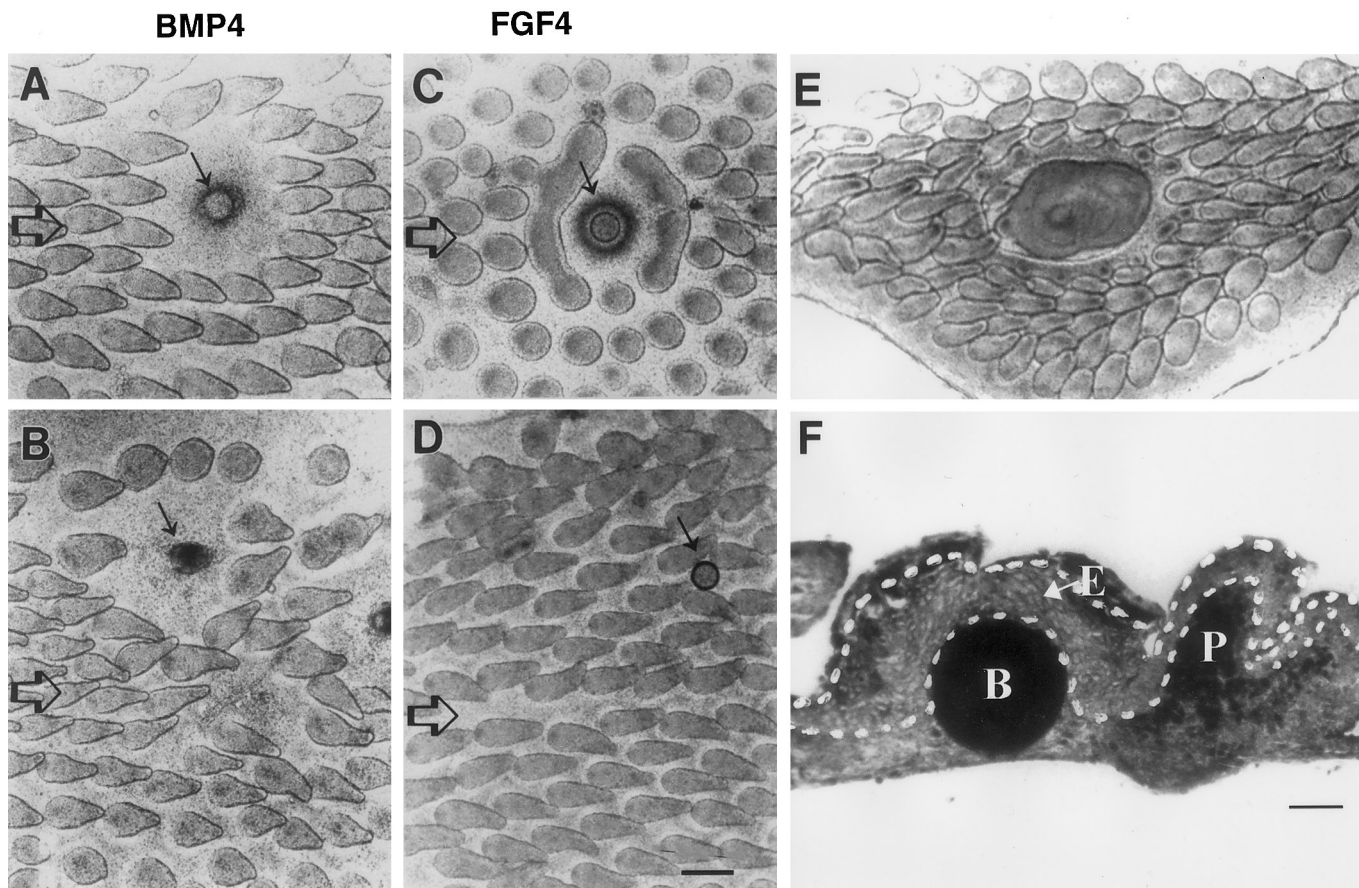
FIG. 3. Local effect of BMP-4- and FGF-4-coated bead and regional competence. (A) BMP-4 bead placed near the midline causes an inhibitory zone around the bead (indicated by arrow). (B) When BMP-4 beads were placed at the lateral region, similar inhibitory effects were observed. (C) FGF-4 beads placed near the midline cause fusion of buds by transforming interbud regions to bud regions. (D) When FGF-4 beads were placed at the lateral edge of early stage explants, there is no apparent effect. The beads are indicated by an arrow and the midline is indicated by an open arrow. (E) SHH beads placed near the midline induce a large feather bud after 4 days in culture. This is similar to the effect with retrovirus-transduced *Shh* (Ting Berreth and Chuong, 1996a). (F) Cross-section of explant with BMP bead. After 3 days in culture with the bead, the epidermis has become thicker and consists of multiple layers of epithelial cells (delineated by white broken lines). The section is lightly stained with H & E and immunochemically with antibody to phosphorylated CREB. PCREB is positive in the bud domain and some regions of the epithelium (Noveen *et al.,* 1995). It is negative around the bead. There are no necrotic or apoptotic changes. B, bead; E, epithelium; P, PCREB. A–E: size bar, 300 μm; F: size bar, 50 μm.

be seen in Figs. 4A′ and 4E and in the ring outside the inhibitory zone of Fig. 4C. They are equivalent to the first phase of FGF-4 expression during feather development (see first paragraph of Discussion). After 2 days of culture, this region either forms new bud domains or buds disappear to become interbud domains. *In situ* hybridization done at this stage shows a distinctive staining pattern of SHH, FGF, and BMP in each feather bud but is not informative (not shown).

The beads in panel Figs. 4A, 4A′, 4B, and 4E have dislodged during the *in situ* hybridization procedure. In Fig. 4A′ and 4E, BMP or SHH is not seen in the location of the bead because the bead itself gradually sinks into the explant, physically displacing tissues after 16 h. This can be appreci-

ated from the cross-section in Fig. 3F. For Fig. 4B, a specimen of 6 h is used; this time the bead has not yet displaced the tissues, so a spot of staining beneath the bead is seen. Figure 4A has a small tissue tear that is not stained. The beads in Figs. 4C and 4D remained in the explant.

Thus, under our experimental conditions, activators can enhance the expression of inhibitors in the surrounding region, while inhibitors can suppress activators, either directly or indirectly, in a negative feedback fashion. We further tested the effect between FGF-4 and SHH. We found that FGF-4 also induced a zone of *Shh* expression around the bead (Fig. 4E). In contrast, there is no apparent induction of *Fgf-4* by SHH (not shown). These results suggest that, *in vitro*, these activators can stimulate the coproduction of

activators and inhibitors within the developing primordia, while inhibitors act to confine the range of activator expression. However, their relationships *in vivo* are likely to be more complex depending on the specific location and/or stages of development in the skin.

DISCUSSION

Initial Expression Sequences: The Continuous Stripe of Fgfs and Shh in the Primary Row and the Later Appearance of Bmps

Feather primordia are arranged periodically. We have asked whether signaling molecules also appear periodically or alternatively whether they start in a continuous fashion and then become punctate as morphogenesis progresses. In the already formed feather buds, we know that *Shh* and *Fgf-4* are in the feather buds. To answer this question, we examined here earlier developmental stages. *In vivo,* feather buds form sequentially, first within the primary row and then propagating laterally (Fig. 5A). We have observed activators (*Shh, Fgf-4*) expressed in two phases. In the first phase, their expression is weak but homogeneously distributed in the primary row. The primary row starts as a continuous stripe. This stripe then breaks into periodic feather primordia showing enhanced *Fgf-4* and *Shh* expression. The interprimordia region becomes completely negative, as if a lateral inhibitory mechanism is acting, and the primordia are gradually sharpened as development progresses. In contrast, the inhibitors *Bmp-2* and *Bmp-4* are not initially present in the primary row. As primordia appear periodically, *Bmp-2* and *Bmp-4* are expressed directly in the primordia and may have a role in lateral inhibition of *Shh* and *Fgf-4.* Thus, different signaling molecules have different modes of appearance.

We also observe a temporal development of competence, across the feather tract, to respond to activators. When stage 29–30 skin explants were cultured with FGF-4-coated beads placed around their midline regions, rings of merged feather buds similar to that described previously (Widelitz *et al.,* 1996) were observed. However, when we placed FGF-4 beads in the lateral regions, there was no effect. In contrast, both BMP-2 and BMP-4 suppressed feather bud formation along the midline or in the lateral regions. The results suggest that, at early stages, regions competent to respond to activators are more restricted than regions competent to respond to inhibitors during development. Later at stage 31, flank regions can respond to FGF-4 too. Thus, we propose that there is a position-dependent competence to respond to activators that first propagates in a posterior to anterior direction and then bilaterally from the midline. The development of this competence serves as a driving force which is manifested as the sequential propagation of feather buds along the midline, then bilaterally. The initiation and direction of propagation of this gradient are body position specific.

Are signals initiated in the epithelium or in the mesenchyme first? From the study here, the epithelium is the tissue that shows distinct molecular expression (Shh, FGF-4) first. However, the early epithelial placodes (stage 29 to about stage 33) are very unstable. Without mesenchyme, placodes and the expression of Shh, Msx1, Msx-2, etc. disappear within 3 h (Chuong *et al.,* 1996). If the epithelium is recombined with a denuded feather mesenchyme, the epithelium, whether previously placodal or interplacodal, is competent to form new placodes. New feather buds reappear in 1 day with molecules appearing in the order of Shh, Wnt-7a (6 h), Notch-1, Delta-1, Serrate-1 (9 h), Msx-1, Msx-2 (18 h), and NCAM and Hox C6 and D4 (>2 day) (Chuong *et al.,* 1996; Chen *et al.,* 1997). The locations of the new buds, however, are determined by the previous dermal condensations where FGFs and BMPs do not disappear. Therefore, appropriate expression of the signaling molecules in feather formation requires intricate epithelial–mesenchymal interactions. The answer to this question is both are the first. Epithelium is first to show an overall competence (the continuous stripe), but this must be ''revised'' by the mesenchyme that is first to set the periodic pattern. Namely, some epithelia originally expressing *Shh* and *FGF* will lose these feather domain molecules and become interbud domains.

BMPs and the Determination of Epithelial Fate

During the stages of feather primordia formation, the epithelium over the feather tract field is originally homogeneous, competent to form either feather placodes or interplacode epithelium. Even after the formation of feather primordia until the early feather bud stage, the epithelia still retain this plasticity and the fate is still reversible. The evidence is that, following the recombination of epithelium and mesenchyme, the previous placodes disappear and new placodes reappear according to the location of the existing dermal condensations. Using DiI labeling, we showed that cells in the previous placode can now become interplacode epithelia and vice versa (Chuong *et al.,* 1996). This suggests that the fate of the epithelia is not determined at this stage. The competent epithelia can respond to the integrated signals resulting from epithelial–mesenchymal interactions to become either feather bud epithelia or interbud epidermis.

In Xenopus, early ectoderm is pluripotential and can become epidermis or neural plate. Activation of the BMP pathway induces the formation of epidermis. In contrast, inhibition of the BMP pathway in Spemann's organizer region, through the direct binding and neutralization by follistatin, noggin, and/or chordin, leads to neural induction (Hemmati-Brivanlou *et al.,* 1994; reviewed in Weinstein and Hemmati-Brivanlou, 1997; Sasai and De Robertis, 1997). In the skin explant cultures, adjacent to the BMP bead we observed a region with multiple epithelial layers that does not form feather buds, suggesting that, under the conditions of our study, the BMP pathway favors the formation of epidermis or future interbud domain. Then the fact that BMP transcripts are specifically expressed in the feather primordium domain appears paradoxical. Indeed in the experimen-
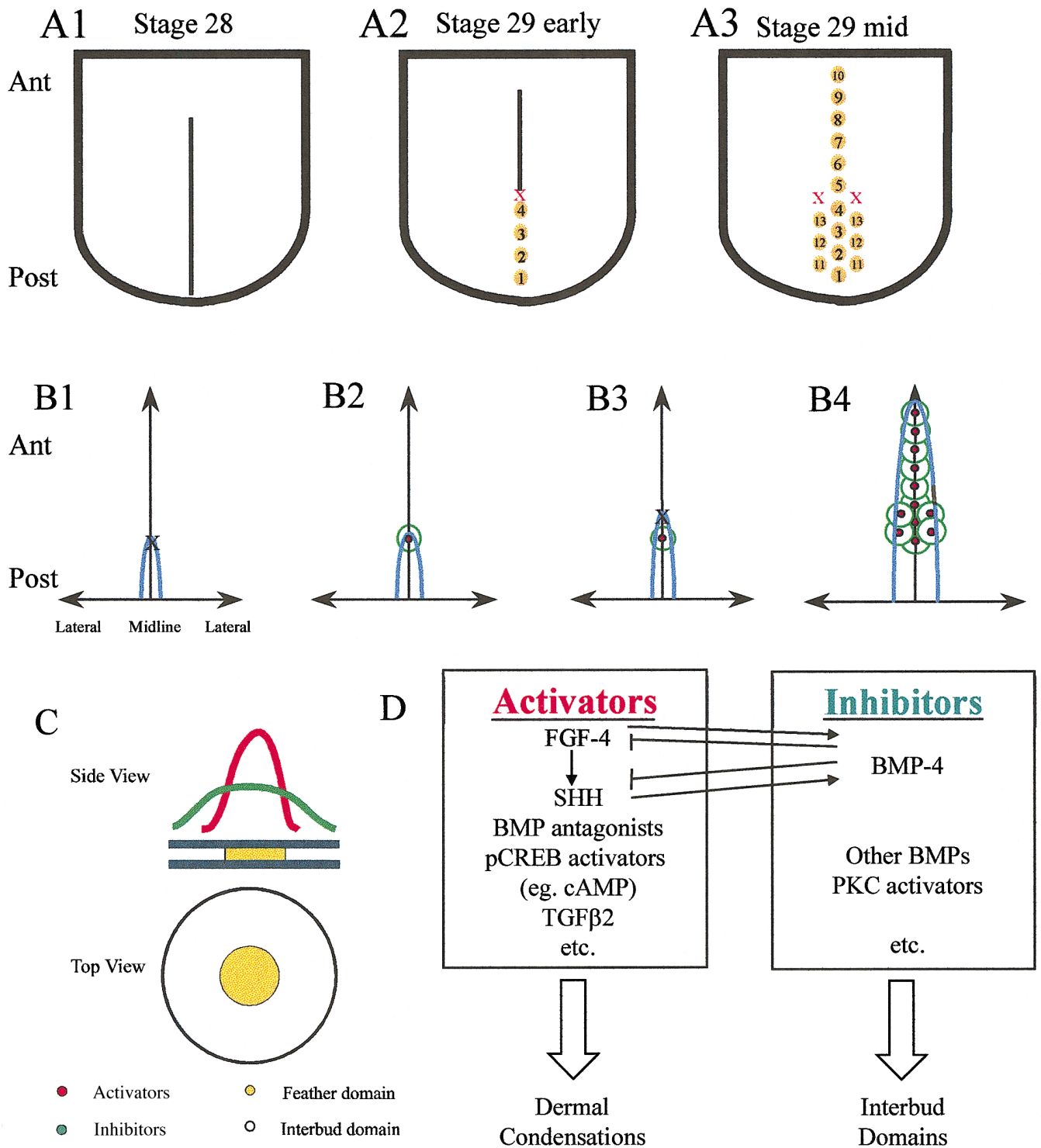
FIG. 5. Working models for feather formation. (A1–A3) Schematic representation of normal feather development. Top view of feather development in lumbosacral region. (A1) Stage 28. Cells are aligned along the midline prior to feather development. This "midstripe" was observed by Stuart *et al.* (1972) and is now shown to be expressing *Shh, Ptc,* and *Fgf-4* (See Figs. 1A, 1D, and 1F). (A2) Early stage 29. Feather bud (brown) development begins from the posterior (Post) end, gradually progressing anteriorly (Ant) and sequentially producing feather buds (numbered circles). At this time, specific distance (~270 μm) and time intervals (~30 min) are maintained between adjacent buds. *Shh* and *Ptc* expression patterns become punctate in regions corresponding to the developing feather buds (see Figs. 1B and 1E).

tal condition immediately adjacent to the BMP-4 bead, there is a rim of induced FGF-4 and SHH expression, which is surrounded by a wider zone of inhibition (Figs. 4C and 4D). This suggests that a high concentration of BMP may play a role in reinforcing the activated regions, while a lower level of BMP works as a negative regulator. Within the bud domain, the activity of BMP can be countered by activators or antagonists of BMPs. For example, our preliminary experiments showed that follistatin is expressed in the feather domain and may act as an antagonist to BMPs (work in preparation). It is possible that the ratio of activators, inhibitors, and antagonists would modulate each other to set up the boundary between a feather bud and the interbud region.

In tooth induction, bead-mediated delivery of BMP can prevent oral epithelium from becoming tooth germs (Neubuser *et al.,* 1997). One recent result is that retroviral mediated ectopic expression of a dominant negative type I BMPR in chicken hind limb buds transformed some scales into feathery scales (Zou and Niswander, 1996). The whole scale was not transformed into a feather bud. Rather, a small feather bud grew out from a portion of the distal margin of the scutate scale. Since the scale is mostly made of multiple layered epidermis (Sawyer *et al.,* 1983), it is possible that a

cluster of transduced cells, missing the activity of the BMP pathway, allows itself to adopt a new fate and form a small feather field within the scale field.

At the cellular level, BMPs have been shown to cause apoptosis, differentiation, reduction of cell motility (Knecht and Harland, 1997), increase of cell adhesion molecules (Lee and Chuong, 1997), etc. At the molecular level, BMPs bind to heterodimers of type I and type II BMP receptors (Koenig *et al.,* 1994; Liu *et al.,* 1995) which are serine/threonine kinases. Binding to the receptor transmits signals through a special group of Mad family members that can act on transcription directly (Kretzschmar *et al.,* 1997). How these molecular and cellular effects are translated histologically to form epidermis or skin appendages is an area that we shall pursue in future research.

A Model for Periodic Feather Pattern Formation

The fact that both the proposed activators (SHH, FGF-4) and inhibitors (BMP-2, BMP-4) are colocalized in the feather primordia regions, rather than having the activators in the primordia and the inhibitors in the interprimordia regions, favors a reaction–diffusion mechanism as proposed by Turing

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(A3) Mid-stage 29. As the primary row propagates anteriorly (black), lateral rows (yellow) begin to develop. The first feather bud of the second row is positioned midway between the first two buds in the first row. Lateral feather bud staining for *Shh* and *Bmp-2* can be seen in Figs. 1C and 1H). (B1–B4) Model depicting several steps along the feather-forming cascade. A computer program based on these principles was prepared in which simulated feather bud formation occurs sequentially in a posterior–anterior direction as well as bilaterally. This model has two components. One is the reaction–diffusion component that acts intrinsically and locally. This is superimposed by the propagation of a ''wave of competence'' that acts at the tract level and may have its origin from body positional information. Together, individual feather primordia are placed periodically and orderly in the competent field. The program is submitted as part of this paper. (B1) During development, a wave of competence factors (blue) traverse the skin in a posterior to anterior direction before feather buds form. Where the competence wave meets the midline stripe (black), a feather initiation site is formed (marked by X). Although the existence of this competence wave is consistent with our results, its molecular nature has not yet been identified. Since the ligands of activators are already present in the midline stripe, we hypothesize this competence to be the ability to respond to the activators (e.g., expression or conformation changes of growth factor receptors or signaling molecules downstream to growth factors). (B2) From the feather initiation site, both activators (red) and inhibitors (green) are released locally and diffuse into the surrounding regions. We presented evidence that SHH and FGFs are activators and BMPs are inhibitors of feather bud formation (Figs. 3 and 4). BMPs are considered long-range morphogens and SHH a short-range morphogen (Lawrence and Struhl, 1996; Lecuit *et al.,* 1996), while the diffusion of FGF may be slowed down by binding to the extracellular matrix (Aviezer *et al.,* 1994). Therefore, in this skin model, the inhibitors are likely to be distributed wider than the activators. A hypothetical cross-section view of the initiation point is shown in A. (B3) As the competence wave continues to travel anteriorly extending outside of the inhibitory field, another feather initiation site is formed (X). Processes C2 and C3 then repeat. This leads to the propagation of feather buds and the conversion from a linear to a periodic pattern, thus forming the primary row. (B4) As the competence wave moves along the midline, it also spreads bilaterally. Therefore, the competence wave moves generally as a half elliptical curve. Processes similar to (C1–C4) repeat in a medial–lateral fashion, thus forming the secondary rows. The simplified computer simulation is presented to show the logic of the model. The following principles are used in programming: IF a point is outside the circle of inhibition AND IF the competence wave has passed this point, THEN insert a new center of activation and a new circle of inhibition. The program is available from Dr. Chuong's web site (http://www-hsc.usc.edu/~cmchuong), or upon request. Further experimental work is required to show their molecular mechanisms. (C) A cartoon to show a hypothetical morphogen distribution based on a reaction diffusion mechanism. In the reaction diffusion mechanism, it is proposed that both activators and inhibitors come out from the same source but diffuse at different rates. Activators (in red) act in short range and are more potent, while inhibitors (in green) act in long range (Koch and Meinhardt, 1994). Away from the center, the ratios of the strength of activators/inhibitors change according to the distance. Immediately adjacent to the diffusion source, activators override the inhibitors, so a primordia domain (in yellow) is established. Further from the center, the inhibitors are higher and the interprimordia space is set. (D) A scheme of the candidate activators and inhibitors compiled from this and previous works is presented. Some molecular relationships are also depicted. The real situation would be more complex and remains to be worked out. One thing to bear in mind is that the relative strengths of the arrows between two molecules probably vary spatially (e.g., see C, from the center of the bud, border of the bud domain, and interbud domain). These differences lead to different fates.

(Turing *et al.,* 1952). According to this model and subsequent modifications (Koch and Meinhardt, 1994; Oster *et al.,* 1983), from the sites of instability, local activators and inhibitors are triggered to diffuse from the same site into the surrounding regions. When the two signals diffuse at different rates, a periodic pattern can form. In this model, activators have a higher potency but a shorter range of action, while inhibitors diffuse further and act over a long range. In the case of feather morphogenesis, feather primordia can initiate from many sites or from one site that then propagates.

FGFs, Shh (activators), and *BMPs* (inhibitors) are localized in the feather primordia region. It has been suggested that FGFs may be concentrated in the immediately adjacent vicinity by binding to the extracellular matrix (Ornitz *et al.,* 1992; Aviezer *et al.,* 1994) and SHH is tethered to the cell membrane through cholesterol (Tabin and McMahon, 1997), which would limit their range of diffusion. BMPs may have a longer range of diffusion but may encounter different antagonists (Weinstein and Hemmati-Brivanlou, 1997). To test the model further it is important to examine the protein distribution of these activators and inhibitors. Here we showed that the distribution of SHH transcripts and proteins is nearly identical. The distribution of FGFs and BMPs should be investigated when antibodies become available. The results here are sufficient to demonstrate that chemical substances play a major role, although this does not rule out that instability can be ascribed to mechanical interactions (Oster *et al.,* 1983). It is possible that growth factors can modulate the expression of adhesion molecules and hence mechanical properties of cells (Edelman, 1992).

For periodic patterning, each primordium needs signals for initiation, expansion, and termination. From our data, it seems that the initiation of feather primordia is first driven by the activators that lead to the formation of many small aggregates. These aggregates then secret local activators as well as local inhibitors. Through positive feedback and lateral inhibition, the competition leads to evenly spaced dermal condensations. During this process, BMP is used to mark the boundary of the bud domain and to set up the interbud domains. Thus, BMPs do not play a role in the initiation, but are essential in setting the periodic pattern.

From these new experimental results, we now propose a model for feather pattern formation that also has its bases on both positional information and a reaction–diffusion system (Turing, 1952; Gierer and Meinhardt, 1972) (Figs. 5A–5C). (1) A feather tract is initiated with global activators expressed in a continuous stripe. (2) Within the tract field, there is a position-dependent gradient specifying competence to form feather primordia (Chuong *et al.,* 1990; Kanzler *et al.,* 1994). For the lumbosacral region of the spinal tract, the gradient has its peak at the posterior end of the midline. (3) As time progresses, the competence propagates from posterior to anterior and then laterally. When the competence allows cells to respond to the activators, a prospective primordia initiation site is set. (4) This event triggers the synthesis and/or release of local activators and local inhibitors. Both factors diffuse into the surroundings, with

inhibitors acting at a longer range than the activators (Fig. 5C). (5) When the effective concentration of the activators drops below that of the inhibitors, the border of the buds is set. Thus, the diameter of the bud may be influenced by the relative strength of local activators and local inhibitors. (Strength is determined by the amount of ligands, receptors, and signal transduction molecules.) (6) When the interaction between the anteriorly advancing competence field and the global activators supersedes the local inhibitors, a new initiation site is formed anteriorly. (7) The anterior–posterior interbud space reflects the relative strength of the global activator and local inhibitor. The stronger the global activator, the smaller the interbud space. (8) When the competence wave spreads gradually to the lateral regions, similar processes are repeated and lateral rows form sequentially.

While FGFs, SHH, and BMPs are ideal candidates for activators and inhibitors, it should be emphasized that there are likely to be other activators, inhibitors, antagonists of activators, antagonists of inhibitors, or modulators acting on different levels of the signaling pathway (Fig. 5D). One example is that protein kinase C is originally all over the mesenchymal cells and then the protein disappears from cells that are becoming part of the bud domain (Noveen *et al.,* 1995). Another example is that CREB is all over the mesenchymal cells, and then only those in the bud domain are phosphorylated (Noveen *et al.,* 1995). A third example is the enrichment of ras pathway components in the feather bud domain when buds start to form (Widelitz *et al.,* 1996). Cells would have to integrate these extracellular and intracellular signals to decide whether to become part of the bud or interbud domains.

We also have begun to explore the relationship of the known signaling molecules in this study. We found that under our *in vitro* conditions, in general, FGF4 and Shh can induce *BMP-4*, while BMP-4 can inhibit *FGF-4* and *Shh* (Fig. 5D). However, the real situation can be more complex. If the activators and inhibitors form a negative feed-back loop, where is the switch point when the dominance of activators is overridden by the inhibitors and the boundary between bud and interbud is set (Fig. 5C)? The relative strengths of activators and inhibitors must vary spatially from point to point in the plane of the skin, and the fate to become part of the bud domain or interbud domain is determined through equilibrium.

Many issues remain to be solved, such as what factors initiate the primary row, what factors establish the anterior–posterior tract competence gradient, what is the molecular nature of competence, how is the size of the bud domain determined, etc. However, this report advances our understanding of periodic patterning by providing several molecular candidates and establishing a framework for periodic feather patterning. It is now possible to test this model further.

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