

## RAPID COMMUNICATION

# Asymmetric Expression of Notch/Delta/Serrate Is Associated with the Anterior–Posterior Axis of Feather Buds

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We studied the roles of Notch, Delta, and Serrate in vertebrate epithelial appendage morphogenesis using feather as a model and found the following. (1) *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are not expressed at the early placode stage and are therefore not involved in the determination of bud versus interbud compartments. (2) From symmetric short buds to asymmetric long buds, *C-Delta-1* and *C-Serrate-1* are expressed in the posterior bud mesenchyme in a nested fashion, while *C-Notch-1* is expressed as a stripe perpendicular to the anterior–posterior (A–P) axis and positioned posterior to the midpoint. (3) Epithelial–mesenchymal recombination with rotation led to the disappearance of these genes followed by their reappearance with new positions appearing to predict their new morphological orientation. (4) Conditions leading to branched buds (e.g., recombination of later buds) show polarized staining patterns before branching occurs. (5) Conditions leading to symmetrical round buds (e.g., treated with the protein kinase A agonist forskolin) suppress expression of all three genes. These results lead us to hypothesize that Notch, Delta, and Serrate are involved in establishing the A–P asymmetry of feather buds. © 1997 Academic Press

## INTRODUCTION

Epithelial appendage morphogenesis involves a series of fate determination processes that progressively transform a homogeneous epithelial sheet into complex structures. We have been using feather development as a model to study this process (Chuong, 1993). At the placode stage, the fate of the epithelium is separated into placodal and interplacodal regions. At the short-bud stage (when the bud height is shorter than the base), the buds protrude out of the surface but remain symmetrical. At the long-bud stage (when the bud height is longer than the base), the buds elongate and become asymmetrical by slanting posteriorly. Therefore during these developmental stages, the presumptive skin is first divided into periodically arranged bud and interbud domains, then the bud domain is subdivided into anterior

and posterior domains. What are the molecules involved in these processes?

Notch and its ligands Delta and Serrate are transmembrane proteins shown to be involved in regulating cell fate commitment in several developmental models (reviewed in Artavanis-Tsakonas *et al.*, 1995; Lewis, 1996). In a field of equivalent precursor cells, activated Notch maintains cells in an undifferentiated state, while cells with inactive Notch are allowed to differentiate. In *Drosophila*, the Notch pathway is involved in neurogenesis through lateral inhibition (Artavanis-Tsakonas *et al.*, 1995) and in wing patterning by the control of position-specific cell proliferation (Speicher *et al.*, 1994). Notch and related genes are expressed in developing (Weinmaster *et al.*, 1991, 1992; Shawber *et al.*, 1996) and mature (Kopan and Weintraub, 1993) rodent hair follicles. These results suggest that Notch may play roles in vertebrate epithelial fate determination. How Notch and related molecules may be involved in the early stages of skin appendage develop-

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ment has not been explored. In this report, we studied their roles in early feather development and found evidence suggesting that the Notch signaling pathway may be involved in anterior–posterior (A–P) axis determination in chicken feather buds.

## MATERIALS AND METHODS

Eggs from SPAFAS (Norwich, CT) were used and staged according to Hamburger and Hamilton (1951). *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* *in situ* probes are from Myat *et al.* (1996). Whole-mount *in situ* hybridization and paraffin section *in situ* hybridization were carried out according to procedures described in Nieto *et al.* (1996). Epithelial–mesenchymal recombination with rotation is described in Chuong *et al.* (1996). Forskolin-treated skin explant culture is described in Noveen *et al.* (1995a). BrdU labeling is described in Noveen *et al.* (1995b).

## RESULTS AND DISCUSSION

### *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* Are Absent in the Placode Stage but Are Expressed in Restricted Patterns in Posterior Compartments When Feather Buds Appear

We used whole-mount and paraffin section *in situ* hybridization to examine the expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* from stage 29 to 36 chicken embryos (Fig. 1). Since feathers start from the midline and propagate laterally, there is a spectrum of developmental stages starting from the lateral edge. *C-Notch-1* is absent in the flat epithelium and placodes and does not appear until the short-bud stage (Figs. 1A–1E). From the top view, *C-Notch-1* is expressed most remarkably as a center stripe (about 70  $\mu\text{m}$  in width, or five to seven cells wide). This stripe is perpendicular to the future A–P axis of the bud, which is still round and symmetrical at this stage (Figs. 1A and 1C). In addition, there is faint expression throughout the posterior half and in a stronger crescent along the posterior margin. This expression is transient. As feather buds elongate, *C-Notch-1* expression becomes weak and is diffusely distributed (Fig. 1B).

*C-Delta-1* is also absent in the placodes and starts to appear at the short-bud stage (Figs. 1A'–1E'). It starts in

the mesenchyme near the posterior edge and extends in the anterior direction, which is manifested as a transition from a crescent to a half-moon staining pattern (Fig. 1A'). As the buds start to elongate, *C-Delta-1* disappears completely (Fig. 1B'). *C-Delta-1* is always negative in the epithelium.

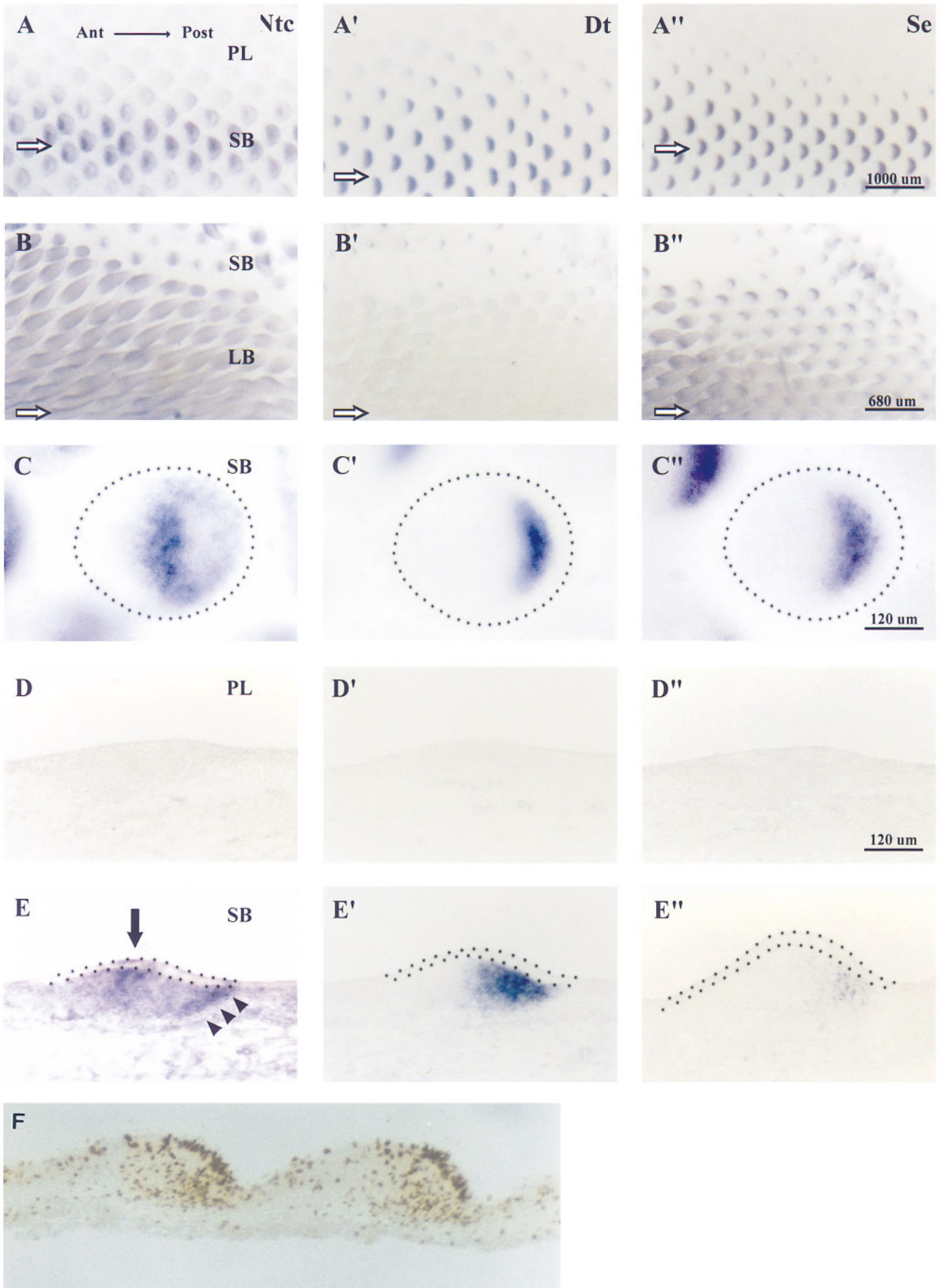
*C-Serrate-1* is also absent until the short-bud stage, then appears in the posterior mesenchyme of short buds in a fashion similar to that of *C-Delta-1* (Figs. 1A''–1E''). From the top, *C-Delta-1* and *C-Serrate-1* appear to have overlapping expression domains at the short-bud stage (Figs. 1C' and 1C''). These particular sections show that *C-Serrate-1* occupies a smaller domain than *C-Delta-1* (Figs. 1E' and 1E''). However, the nested relationship shifts during development and comparison of precise compartment borders will depend on future double *in situ* hybridization. At the long bud stage, *C-Serrate-1* becomes localized to the proximal posterior region of the bud (Fig. 1B'').

The absence of these three genes at the early placode stage precludes the possibility that *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are involved in the initiation of feather primordia. However, it is possible that other forms of Notch, Delta, and Serrate (Weinmaster *et al.*, 1992; Myat *et al.*, 1996; Shawber *et al.*, 1996) may be involved at the early stages.

Section *in situ* hybridization showed that *C-Delta-1* and *C-Serrate-1* are restricted to the mesenchyme (Figs. 1E' and 1E''). *C-Notch-1* is mainly in the mesenchyme with weaker staining in the epithelium (Fig. 1E). The molecular subdivision of feather buds into several anterior–posteriorly arranged compartments by Notch, Delta, and Serrate strongly suggests that they are involved in establishing A–P asymmetry. These compartments may then show characteristic cellular behavior such as differential cell proliferation that can contribute to the asymmetric shaping of the buds. For example, if cells in the posterior bud proliferate faster than cells in the anterior bud, there will be more expansion in the posterior domain and the bud will slant. In accord with this hypothesis, we observed preferential distribution of S-phase cells in the posterior feather bud epithelia and mesenchyme using BrdU labeling (Fig. 1F; also see Desbiers *et al.*, 1992).

Several experimental conditions can perturb feather A–

**FIG. 1.** Expression pattern of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* in developing feather buds. *In situ* hybridization of chicken dorsal skin explants. *C-Notch-1* (A–E), *C-Delta-1* (A'–E') and *C-Serrate-1* (A''–E''). Feather buds from placode stage (PL), short-bud stage (SB), and long-bud stage (LB) are shown. Anterior is to the left. *Whole mount*: A–A'', stage 33. B–B'', stage 36. White arrows in A–B'' line up with the middle row of each skin explant. Note there is a maturation gradient from the midline to the lateral rows. C–C'', enlarged short buds, with dotted lines placed outside the epithelial border of the bud. Note feather primordia of different stages show different expression patterns. Most dramatic is the mid *C-Notch-1* stripe and the posterior mesenchymal nested pattern of *C-Delta-1* and *C-Serrate-1* at the short-bud stage. *Sections*: D–D'', placode stage. *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are absent. E–E'', short-bud stage. *C-Notch-1* is mainly in the mesenchyme and weakly in the epithelium. *C-Delta-1* and *C-Serrate-1* are only in the mesenchyme. In the *C-Notch-1* panel, the straight arrow indicates the midline stripe, and the arrowheads point to the posterior crescent. The dotted lines mark the epithelial boundary over a feather bud. *BrdU labeling*: F, populations of S-phase cells are preferentially localized in the posterior epithelium and mesenchyme of the short feather buds.



P axial orientation. If molecules of the Notch pathway are involved in determining the A–P axis, their expression should be polarized before altered morphological asymmetry.

***C-Notch-1, C-Delta-1, and C-Serrate-1 Expression Is Regulated by Epithelial and Mesenchymal Interactions and the New Polarized Expression Pattern Precedes Morphological Asymmetry Following Recombination***

**Rotated buds.** Epithelium and mesenchyme from skin explants can be separated and recombined. Old feather buds will disappear and new buds will form. To test the origin of the orientation activity, we can rotate the relative positions of the epithelia and mesenchyme, recombine them, and then culture the recombined explants. When this was done on skins from stage 29 to 33, the locations of the new feather buds were in accord with the original dermal condensations, but their A–P orientation was in accord with the original epithelium (Novel, 1973; Chuong *et al.*, 1996). We examined the expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* following a 90° rotation (Figs. 2A–2B"). Three hours following the recombination, *C-Notch-1* and *C-Delta-1* have disappeared, and the *C-Serrate-1* transcript is reduced and will disappear. Naked mesenchyme (to the right of the dashed lines in Figs. 2A–2B") also lose their *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* transcripts but, surprisingly, at a slower rate. These results suggest that the maintenance of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* requires the interactions between epithelia and mesenchyme, and that epithelium not directly above the Notch stripe may have a negative effect on the mesenchymal expression of Notch.

Thirty hours after recombination, when the buds are still round and symmetrical, *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are expressed asymmetrically. For *C-Notch-1*, the central stripe is now reoriented to be perpendicular to the new cephalic–caudal orientation of the epithelium. Similarly, *C-Delta-1* and *C-Serrate-1* reappeared in a polarized

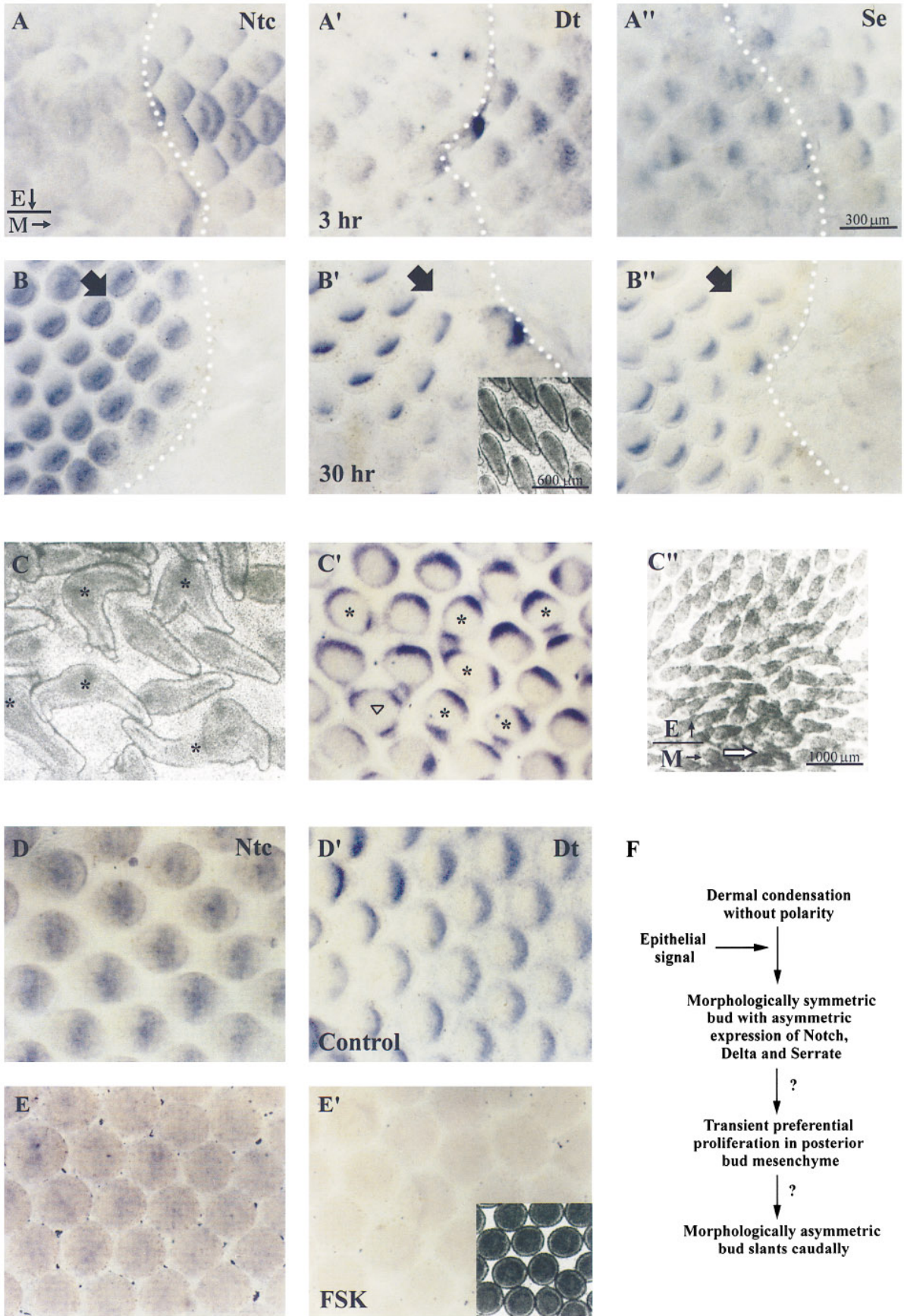
fashion. In 72 hr, the regenerated buds elongate and become morphologically asymmetric (Fig. 2B' inset). The A–P axis is set in the direction as predicted by the positions of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1*.

**Branched buds.** When recombination/rotation was carried out on explants from stage 34 embryos, many branched feather buds formed (Fig. 2C). We then examined the expression of these genes under this experimental condition. At 10 hr after recombination, we observed bipolar and even tripolar expression of *C-Delta-1* in the round buds (Fig. 2C'), as a prelude for the subsequent branched feather bud formation. When recombination/rotation was carried out on skins from stage 35 embryos, the mesenchymal orientation of the more mature buds (those flanking the midline of the explant) becomes resistant to change; however, the lateral mesenchyme is still receptive to the orientation signals from the epithelia (Fig. 2C"). At stage 35, long feather buds have formed in regions flanking the midline of the explant, while the rest of the skin is mainly composed of short feather buds. This suggests that dermal condensations up to the short-bud stage are flexible and can respond to epithelial signals to set up new orientations. As they mature into long feather buds, the flexibility of the mesenchyme is gradually lost and the orientation signals shift from the epithelium to the mesenchyme. In the middle of this transition, when the epithelium and the mesenchyme have about equal orientation signals, branched buds form.

***Protein Kinase A (PKA) Agonists Lead to Round Symmetric Buds That Lack the Expression of C-Notch-1, C-Delta-1, and C-Serrate-1***

**Symmetric buds.** PKA agonists such as cAMP and forskolin can enhance dermal condensations and inhibit feather bud elongation. The phenotype is discrete short round buds (Noveen *et al.*, 1995a). If the Notch pathway is involved in setting up the A–P axis, we predict that there should be a suppressed or diffuse expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* in the round feather buds in this type of skin explant. Indeed we observed the normal stripe

**FIG. 2.** Expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* in experimentally manipulated feather buds. **Rotated buds:** A–B", epithelial (E)–mesenchymal (M) recombination with 90° rotation was performed on skin from stage 33 embryos. Whole-mount *in situ* hybridization using *C-Notch-1* (A, B), *C-Delta-1* (A', B'), and *C-Serrate-1* (A", B") probes on explants fixed at 3 (A–A") and 30 hr (B–B") after recombination. Thin arrows point to the posterior end of the epithelium or mesenchyme. The dashed lines mark the border of the recombined epithelium. Regions to the right of the line are naked mesenchyme. Broad arrows point to the posterior of regenerated feather buds when they elongate in 3 days (inset in B'). Note the disappearance and reappearance of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1*. **Branched buds:** C–C", E–M recombination with rotation was carried out on skin from stage 34 embryos. C, phase contrast photograph of the explant after 5 days of culture shows many branched feather buds (\*). Whole-mount *in situ* hybridization using *C-Delta-1* probe (C', 10 hr after recombination) show bipolar (\*) and tripolar (∇) expression. C", E–M recombination with 90° rotation was carried out on skin from stage 35 embryos. Mesenchyme flanking the midline (white arrow) are more mature and maintain original orientations. Mesenchyme toward the lateral edge (top) are still flexible and follow the orientation of the epithelia. **Symmetric buds:** D–E", skin explants were not treated (D, D') or treated (E, E') with the PKA agonist, forskolin (20 μM). The explants were hybridized with *C-Notch-1* (D, E) and *C-Delta-1* (D', E') 18 hr after culture. Note the disappearance of *C-Notch-1* and *C-Delta-1*. The buds remain round and short 5 days after culture (inset in E'). **A working model:** F, events are listed in time sequence. Question marks indicate that a causal relationship is not established. The nature of epithelial A–P positional information remains to be determined and may involve PKA, Wnt 7a, gap junction communication (Serras *et al.*, 1993), or other molecular activities.



and posterior expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* in control explants, but a lack of their expression in forskolin-treated explants 18 hr after culture (Figs. 2D–2E' and not shown). Five days later, while control buds have elongated (similar to Fig. 2B' inset), the forskolin-treated buds are still round and short (Fig. 2E', inset). Thus, the polarized expression of *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* at the short-bud stage appears to predict the elongation and the axial orientation of this elongation at the long-bud stage.

The phosphorylated cyclic AMP-responsive enhancer binding protein (pCREB) can be detected by antibody staining and used as an indicator of PKA activity. pCREB is preferentially expressed in the anterior short bud (Fig. 1C of Noveen *et al.*, 1995a) at about the time *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are preferentially expressed in the posterior mesenchyme. Thus it is possible that activation of PKA may downregulate Notch, Delta, and Serrate expression in the anterior bud. Speculatively, we may view the cAMP-treated buds as completely "anteriorized" by eliminating the Notch pathway and the posterior compartment.

This report sets the base to pursue many interesting issues regarding the establishment of A–P asymmetry in feather buds. It is apparent now that this event does not occur until the transition from short-bud to long-bud stage. A review of previous data showed that NCAM and Hox C6 are expressed all over the bud mesenchyme at the short-bud stage, and only become localized to the anterior mesenchyme in long buds when bud morphology is asymmetrical (Chuong and Edelman, 1985; Chuong *et al.*, 1990). Similarly, Sonic hedgehog is symmetrically expressed in the central placode at the short-bud stage, and only becomes localized to the posterior placode at the long-bud stage (Ting-Berret and Chuong, 1996). Notch, Delta, and Serrate are unique because they are already polarized when buds are still round. Two other molecules are asymmetrically expressed at a comparable early stage. One is pCREB in the anterior mesenchyme (Noveen *et al.*, 1995a). The other is Wnt 7a in the posterior placode epithelium (Chuong *et al.*, 1996). Whether these molecules are involved in regulating the expression of Notch, Delta, and Serrate *in vivo* and which molecule(s) is the initiator of A–P axis determination remain to be determined.

Supposing that Notch, Delta, and Serrate are involved in establishing the A–P axis, what may be the mechanism? In the imaginal wing disc of *Drosophila*, Serrate has been shown to mediate position-specific cell proliferation and contributes to the control of dorsal/ventral patterning (Speicher *et al.*, 1994; Diaz-Benjumea and Cohen, 1995). In the short feather buds, BrdU labeling is originally homogeneously distributed, but later shifts to the posterior region (Fig. 1F; Desbiens *et al.*, 1992; Noveen *et al.*, 1995b). One compelling hypothesis is that differential growth of the anteriorly–posteriorly arranged compartments can lead to an asymmetric morphology (Figs. 1F and 2F). A higher proliferation potential may be maintained in cells with an activated Notch pathway. To test this hypothesis, in the future we will test whether feather symmetry is altered by misex-

pressing molecules in the Notch pathway with retroviral gene transduction. We will also analyze cell lineage and trafficking in the forming buds with lineage tracer labeling.

It is surprising that *C-Notch-1*, *C-Delta-1*, and *C-Serrate-1* are not involved in the early specification between primordia and interprimordial regions. However, there are multiple members of Notch and related signaling molecules (Weinmaster *et al.*, 1992; Myat *et al.*, 1996; Shawber *et al.*, 1996) that may act at different stages during feather morphogenesis. Further work is required to determine how the expression domains of these molecules are lined up (juxtaposed, overlapped, or apart?) and interpreted by local cells. The sharing of Notch signaling molecules in *Drosophila* and avian epithelial appendage morphogenesis further demonstrates the fundamental nature of this signaling mechanism. Elucidation of these processes will advance our understanding in the intricate patterning of feathers.

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