

Mouse Notch: Expression in Hair Follicles Correlates with Cell Fate Determination

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Abstract. Many vertebrate tissues, including skin, are known to develop as a consequence of epithelial–mesenchymal interactions. Much less is known about the role of cell–cell interaction within the epithelial or the mesenchymal compartments in morphogenesis. To investigate cell–cell interactions during skin development, and the potential role of the Notch homolog in this process, we cloned the mouse homolog of Notch (mNotch) and studied its expression pattern, starting as early as mesoderm formation. The novel application of double-labeled in situ hybridization in vertebrates allowed high resolution analysis to follow the fate of mNotch expressing cells directly. In comparison with the distribution of Id mRNA, analysis confirmed that in the hair follicle high levels of mNotch are expressed exclusively in the epithelial compart-

ment. Hair follicle matrix cells start expressing mNotch as different cell types become distinguishable in the developing follicle. mNotch mRNA expression persists throughout the growth phase of the follicle and maintains the same expression profile in the second hair cycle. The cells in the follicle that undergo a phase of high level mNotch expression are in transition from mitotic precursors to several discreet, differentiating cell types. Our observations point out that both in time (during development) and in space (by being removed one cell layer from the dermal papilla) mNotch expression is clearly separated from the inductive interactions. This is a novel finding and suggests that mNotch is important for follicular differentiation and possibly cell fate selection within the follicle.

THE vertebrate skin is an example of a complex tissue initially formed as a result of inductive epithelial–mesenchymal interactions. A variety of epithelial structures and cell types are generated: protective stratified epithelium, glands, hairs, feathers, etc. (Sengel, 1976; Sengel, 1990). In contrast to the focus given to inductive (heterotypic) interactions which initiate the process, less attention has been given to the potential role of cell–cell interactions within the epithelial or the mesenchymal compartments (homotypic interactions) in morphogenesis.

Homotypic cell–cell interactions play a fundamental role in establishing separate identities for cells of equivalent developmental potential in *Drosophila melanogaster* and in *Caenorhabditis elegans*. Members of the Notch/*lin-12* family play a key role in mediating such interactions. In *D. melanogaster*, for example, Notch acts to resolve a cluster of neurogenesis-competent cells into epidermal cells and neurons (e.g., see reviews by Artavanis-Tsakonas and Simpson, 1991; Heitzler and Simpson, 1991). Notch is also involved in determination of oocyte polarity and somatic follicle cell specification (Ruohola et al., 1991) and in the establishment of multiple cellular identities during eye development (Cagan and Ready, 1989). Evidence from genetic and biochemical studies in *D. melanogaster* suggest that Notch functions in a cell autonomous fashion, perhaps as a receptor for specific ligands (Fehon et al., 1990; De Celis et al., 1991;

Fehon et al., 1991; Heitzler and Simpson, 1991; Rebay et al., 1991). Although apparently involved in choosing between two alternative fates, Notch/*lin-12* proteins do not establish cell fates per se, rather they mediate intracellular signaling such that cells select one of two alternative fates (reviewed by Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992).

Sequence information reveals that family members share several structural features: a large extracellular domain containing eleven (*glp-1*), 13 (*lin-12*; Yochem and Greenwald, 1989), or 36 (Notch) EGF-like repeats followed by three Notch/*lin* repeats (LNR) and a transmembrane domain. The intracellular domain contains six repeats similar to *cdc10/swi6*/ankyrin motifs thought to be involved in protein–protein interactions (LaMarco et al., 1991), as well as the “opa” and “pest” sequence motifs. The “pest” motif is thought to control protein stability (for review, Greenwald and Rubin, 1992). The structure of Notch/*lin-12* is consistent with its proposed role as a signalling receptor. Homologous proteins have been cloned in *Xenopus* (Coffman et al., 1990), rat (Weinmaster et al., 1991), human (Ellisen et al., 1991), and mouse (this report, and Franco del Amo, 1992). The striking degree of conservation between species prompted the suggestion that vertebrate homologs play a similar role in cell–cell interactions during vertebrate development (Coffman et al., 1990; Weinmaster et al., 1991).

We are interested in exploring the possibility that the vertebrate Notch homolog participates in homotypic interactions during skin development, starting as early as mesoderm formation. Previous studies of skin development have found it to be a multi-step process starting during gastrulation. Appendage (hair or feathers, glands) formation in embryonic skin involves the following sequence of histological changes: ectodermal cells at specific locations become columnar (formation of epidermal placode). After deposition of a collagen lattice under the epidermis, mesenchymal cells under the epidermal placode form mesenchymal condensations that will become dermal papillae, the mesenchymal component of hair and feathers (Wessells, 1965; Stuart and Moscona, 1967; Stuart et al., 1972; Holbrook et al., 1988; Kopan and Fuchs, 1989). The inductive influence of dermal papilla cells causes epithelial placode cells to divide and invade the dermis, forming buds of epithelial cells (Oliver and Jahoda, 1988). Genetic evidence and results of transplantation experiments suggest that an ectodermal signal may actually initiate this process since such a signal seems to be required for the mesenchyme to display its inducing potential (for review see Goetinck et al., 1980; Sengel, 1976). The ability of mesoderm to induce appendages in specific positions is initially acquired just ahead of the visible wave of appendage morphogenesis, but after several rows of appendages have formed all the mesoderm seems to have acquired inductive potential (Davidson, 1983; Linsenmayer, 1972). The position, number, and morphology of appendages induced by the mesoderm in a given area is dependent on the rostrocaudal origin of the mesoderm (for review see Hardy, 1992; Sengel, 1976, and references therein). Induction of the overlying ectoderm by dermal papillae is followed by appendage morphogenesis, which in the hair follicle includes the generation and differentiation of seven specific cell types organized symmetrically about a central axis (see Fig. 1; for example see Feitz et al., 1990; Kopan and Fuchs, 1989). In mammals, hair follicles degenerate, the dead hair fiber remaining attached at its base. A new cycle of induction-morphogenesis-differentiation followed by degeneration repeats itself throughout adulthood. This phenomenon is known as the hair cycle.

In this paper, we report the mNotch mRNA expression patterns during skin development. We chose to examine this question because of the parallels between the generation of cell types in the mammalian skin versus the *D. melanogaster* ectoderm and observations made in rat whiskers (Weinmaster et al., 1991) that suggested involvement of Notch in reciprocal epithelial-mesenchymal interactions. mNotch mRNA was detected in hair follicles in cells preparing to differentiate into the individual cell types of the hair shaft and inner root sheath. Our observations point out that both in time (during development and during the hair cycle) and in space (by being removed one cell layer from the dermal papilla) mNotch expression is clearly separated from the inductive interactions per se. Thus, our findings demonstrate that mNotch is positioned to play a role during acquisition of cell fate in the hair follicle.

Materials and Methods

Staging of Mouse Embryos

CD-1 mice were kept under a 12/12-h dark/light regiment. Matings were

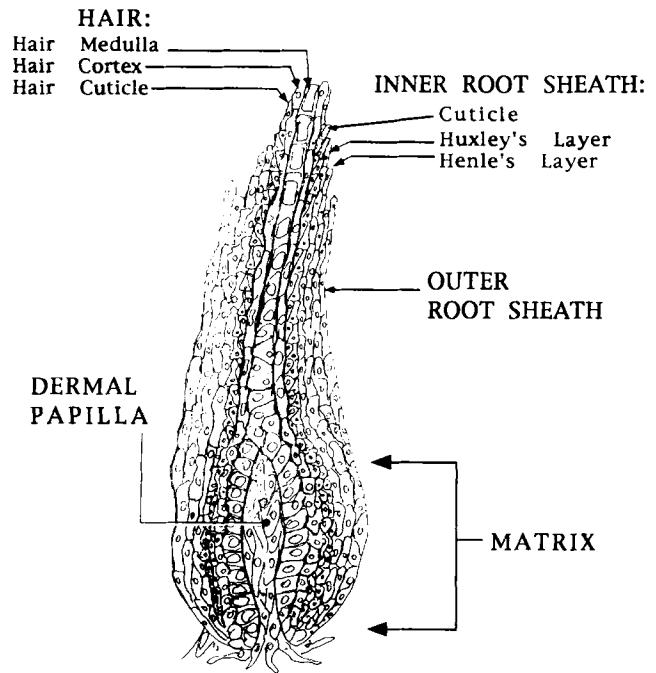


Figure 1. Differentiated cell types within the mouse hair follicle. The hair follicle forms as a bulb around the specialized mesenchymal cells, i.e., dermal papilla. The relatively undifferentiated matrix cells are derived from the embryonic basal layer of developing mouse skin, and give rise to concentric rings of differentiated cell types. Cell layers are described from the axis of symmetry out. The hair consists of the medulla, the cortex, and the cuticle of the cortex. It is surrounded by two root sheaths. The inner root sheath is composed of three cell types: the inner root sheath cuticle, Huxley's and Henle's layers; the outer root sheath is composed of few layers of a single cell type. The hair and inner root sheath cells extend to the dermal papilla, where they are active mitotically and give rise to cells which migrate upward in single cell layers of distinct differentiative pathways. The outer root sheath cells do not come into direct contact with the dermal papilla. They are contiguous with the basal epidermal layer, but they are distinct and self-propagating.

set late afternoon and vaginal plug inspection was done the next morning. For timing purposes, day 1 of pregnancy, or day 1 post coitum (pc)¹, was the day on which the plug was detected.

Cloning and Mapping of mNotch

For the cloning of a short fragment containing the cdc10 repeats of mouse Notch, two degenerate PCR primers were made for conserved amino acids between the only mammalian Notch sequences available at the time: human (Adams et al., 1991) and frog (Xotch, Coffman et al., 1990) homologs. For the 5' primer the amino acid sequence chosen was TKKFRFEE (GCTGGATCCACNAARAARTTYMGNTTYGARGA) and for the 3' primer the amino acid sequence chosen was MDRLPRD (GCTCTCGAGRTCNCCKNGGNARNGKRTCCAT). mRNA was isolated from day 13 pc or day 11 pc embryos and RT/PCR reactions performed as previously described (Rupp and Weintraub, 1991). 2 µg of each primer were used in each 50-µl reaction. The cycle parameters were: 94°-, 60°-, and 72°- 1 min each for 42 cycles. The PCR products were digested with BamHI and XhoI (New England Biolabs Ltd., Beverly, MA) under standard conditions, gel purified fragments of ~900 bp were cloned into BamHI/XhoI digested plasmid vector bluescript KS+ (Stratagene, La Jolla, CA), and the nucleotide sequence of several clones was determined. Clones containing sequence similar to the *Xenopus* and *D. melanogaster* Notch were kept (plasmid pMotch). The

1. Abbreviations used in this paper: AlkPh, alkaline phosphatase; ISH, in situ hybridization; pc, postcoitum; peg, polyethylene glycol; pp, postpartum.

mRNA obtained for the RT/PCR was used to make a cDNA library with the SuperScript lambda cloning kit (GIBCO/BRL). To maximize recovery of cDNA, size selection was achieved by polyethylene glycol (PEG)-induced precipitation (9% PEG, 500-bp cut off) (Lis, 1980). Plaques were screened with a ³²P-labeled, PCR generated probe from pMotch. One partial cDNA clone (called pksMotch), was recovered and its nucleic acid sequence was determined. This cDNA clone contained most of the intracellular domain of mNotch, starting at a NotI site and extending into 3'-untranslated sequence. pMotch extended beyond that site and a combined clone was constructed after the nucleic acid sequence of 150-bp overlap proved to be identical. Fig. 2 A presents a comparison between the rat and the mouse sequences. Fig. 2 B presents a comparison between mNotch and the two rat Notch genes at the amino acid level.

Chromosomal Location of mNotch

Mouse genomic library (Sau3aI partially digested genomic DNA cloned into lambda Dash vector; Stratagene) was screened with ³²P-labeled, PCR-generated probe from pMotch, and a clone containing a 20-kb fragment was recovered. Restriction mapping, partial sequence analysis and PCR amplification with probes derived from cDNA sequence revealed that this clone (G3) contained the carboxy-terminal region of mNotch corresponding to our cDNA and identified several intron-exon boundaries. The chromosome on which mNotch is located was determined using hybrid cell lines (hybrids F[2.8]D, FF4-3a, and 17T-1; generously provided by Dr. K. Fournier, Fred Hutchinson Cancer Research Center, Seattle, WA), with previously determined mouse chromosome complement (Tapscott et al., 1988). PCR primers spanning a small intron were used to identify the hybrid DNA containing the mNotch gene (5'-GCTGCTGACCTGCGCATG-3' and 5'-ATC-TTCTTCTTCTTCACTG-3'). DNA amplification was done in standard conditions. The cycle parameters were: 94°, 1 min, 60°, 30 s, and 72°-1 min for 35 cycles.

Southern Blot Analysis

Mouse genomic DNA and genomic DNA clone (G3) were digested with NotI, Sac I, and HindIII, (all enzymes cutting twice within our genomic clone and once within our cDNA). Southern blots containing G3 DNA run side by side with digested total genomic DNA were hybridized with a PCR probe generated from our cDNA (same primers that were used for the cell hybrid analysis). This probe was contained within the digested fragments. The hybridizing bands were identical under stringent washing conditions and consistent with a single gene copy.

In Situ Hybridization

The plasmids described above and pKSV15 (wool type I keratin; Bertolino et al., 1990; Kopan and Fuchs, 1989) were labeled with either digoxigenin 11 UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) or biotin 11 UTP (Sigma Immunochemicals, St. Louis, MO) to generate sense and antisense RNA probes. Probes were hybridized to an average size of 100-110 nucleotides by sodium carbonate treatment at 60°C. Whole-mount in situ hybridization was a modification of a previously described protocol (Harland, 1991). Fixation in 4% paraformaldehyde varied between 2 h and overnight with no detectable differences in signal. The following modifications were needed to reduce nonspecific background. Rehydrated mouse embryos were treated for 20 min in ice cold 20% acetic acid (J. T. Baker, Phillipsburg, NJ) and subsequently washed in PBS until the wash was at pH 7.4. All steps were increased in time to permit diffusion and equilibration in the larger mouse embryos. Sheep affinity-purified anti-digoxigenin antibody coupled to alkaline phosphatase (AlkPhs; Boehringer Mannheim Biochemicals) had to be absorbed for 1 h (on ice) with homogenized, prehybridized day 16-pc embryos. Before use, the mixture was spun down and the supernatant used for immunohistochemistry.

Double Label In Situ Hybridization and Immunohistochemistry

The protocol we used diverged from ³⁵S-UTP based in situ hybridization protocols in the following detail: tissue sections were adhered to aminoalkylsilane-treated glass slides (Rentrop et al., 1986). Both probes were hybridized to the tissue sections simultaneously. The hybridization conditions we used were: 50% formamide (EM), 5× SSC, 1 mg/ml Torula RNA (Sigma Immunochemicals), 100 µg/ml heparin (Sigma Immunochemicals), 1× Denhart's, 0.1% Tween 20 (Sigma Immunochemicals), 0.1% CHAPS (Sigma Immunochemicals), 1 µg/ml of each probe. After, silane-

treated coverslips were placed over the sections, slides were placed under paraffin oil at 55°C overnight to permit hybridization. RNase A digestion was followed by two 30 min room temperature 2× SSC washes and two 0.1× SSC washes at 60°C. Detection of probes was done sequentially. Blocking agents used were BSA (2%) and goat serum (5%) in PBS (BSA/PBS). The blocking solution was replaced with 1:1,000 dilution of a sheep affinity-purified anti-digoxigenin antibody coupled to AlkPhs (Boehringer Mannheim Biochemicals) in BSA/PBS and incubated at room temperature for 1 h. Sections were washed five times in PBS (15-min each) and stained according to Harland (1991). Staining was visually monitored with an inverted microscope (Nikon Instruments, Melville, NY). Signal for mNotch was obtained within 16 to 24 h. The staining reaction was stopped by incubating the sections in 4% paraformaldehyde/PBS for 30 min. After fixation, sections were washed three times in PBS (15-min each). After 15 min in blocking solution, the sections were incubated in 1:2,000 streptavidin-β-galactoside conjugate (Boehringer Mannheim Biochemicals) in BSA/PBS. Sections were stained in PBS containing 1 mg/ml X-Gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂. The reaction was allowed to develop for 24-36 h. To compare the sensitivity and fidelity of the same probe with different detection protocols, adjacent skin sections were hybridized with either antisense digoxigenin-labeled keratin probe or a biotin-labeled keratin probe. The results showed that the same cells were detected whether a single probe or two probes were used, irrespective of detection method, although the AlkPhs was more sensitive (data not shown). We used the more sensitive method for the less abundant mRNA. Thus the blue signal served as an internal reference for both the onset of cortical differentiation and a marker of cortical cells within the follicle. Signal was clearly visible in 4 h. Comparison between day 26 postpartum (pp) hybridized with either sense RNA control or an antisense probe showed signal only in the developing follicle hybridized with antisense probe.

Results

Cloning of mNotch and Its Chromosomal Location

To generate probes for in situ hybridization, degenerate PCR primers, based on comparison of sequence information from frog and human Notch homologs (Adams et al., 1991, see Materials and Methods), were designed and used to clone a 900-bp fragment from day 13 pc mouse embryo mRNA. Sequence analyses identified the fragment as the cdcl0 domain of the mouse Notch homolog, mNotch. This fragment was then used to screen a day 13-pc cDNA library and a clone corresponding to the intracellular portion of mNotch was recovered. After the sequence of a long overlap proved to be identical, the cDNA clone (pksMotch) and the PCR clone (pMotch) were combined at a NotI site.

Since several Notch homologs are suspected to exist in the mouse (Ross, M. E., and N. Heintz, 1991. *Soc. Neurosci. Abstr.* 17:707) and a second Notch gene has been recently reported in rats (Weinmaster et al., 1992) and humans (Stifani et al., 1992), sequence comparison between rat Notch (Weinmaster et al., 1991), and our clone was performed. The sequences are 93% identical at the nucleotide level (Fig. 2 A shows the alignment of the cdcl0 repeats included in our pMotch plasmid). Sequence comparison at the amino acid level between rat Notch I (Weinmaster et al., 1991), rat Notch II (Weinmaster et al., 1992), and mNotch (Fig. 2 B) confirmed that mNotch is the homolog of rat Notch I.

Next, we attempted to find the chromosome containing the gene coding our cDNA. After cloning a genomic fragment corresponding to our cDNA, we identified by sequence analysis several exon-intron boundaries (see Materials and Methods). To determine the chromosomal location of the mouse Notch homolog we used specific PCR primers spanning a small intron differentiating between the mouse and the rat genes. These primers were used to screen somatic cell hybrids. Only F(2.8)D rat cells, containing mouse chromo-

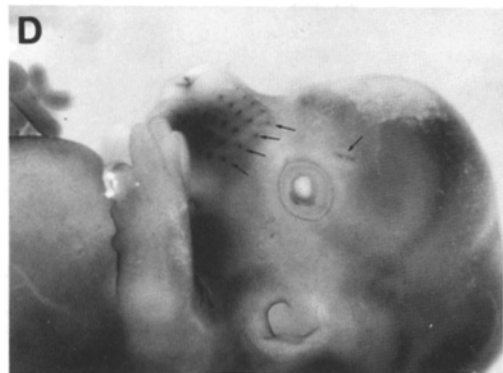
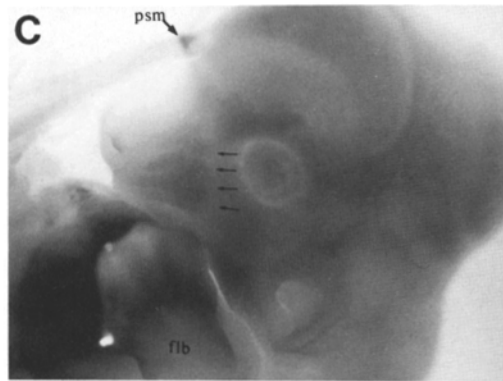
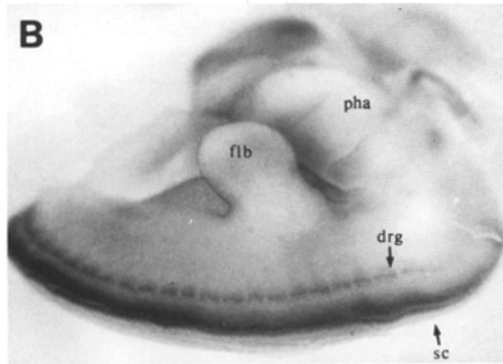
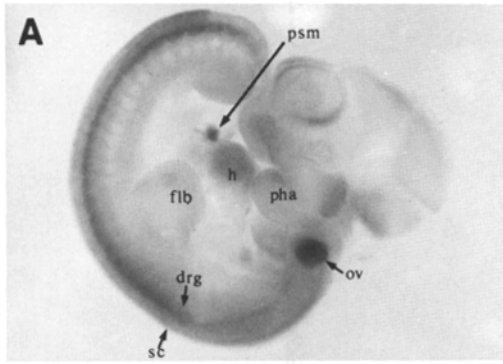


Figure 3. Localization of mNotch mRNA in day 11–14 pc embryo. Embryos were hybridized with either sense (not shown) or antisense (A–D) mNotch RNA probes containing digoxigenin 11 UTP. After RNase digestion and washes, the probe was detected by alkaline-phosphatase-conjugated anti-digoxigenin antibodies and a colorimetric alkaline-phosphatase reaction. (A) At day 11 pc mNotch expression is absent from pharyngeal arches (*pha*) and lateral mesoderm. Signal is detected in auditory vesicle (*ov*), dorsal root ganglia (*drg*), spinal cord (*sc*), and presomitic mesoderm

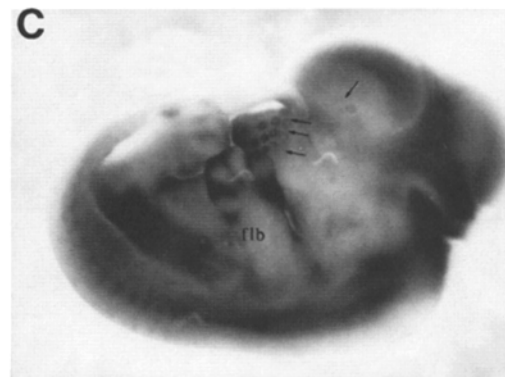
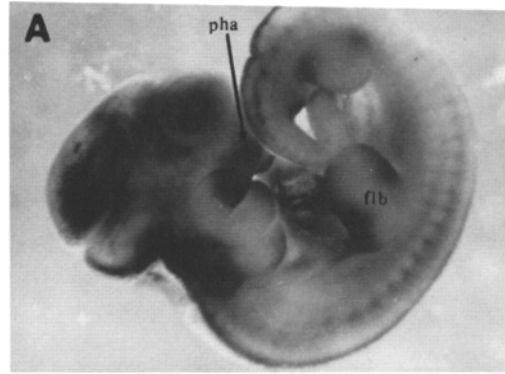
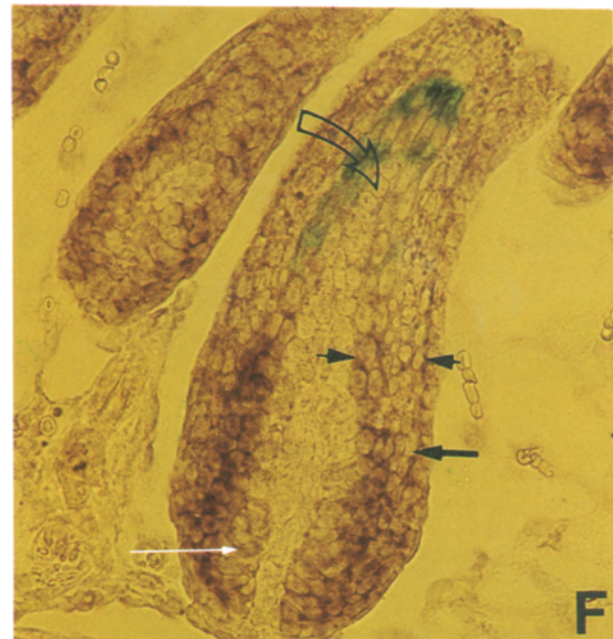
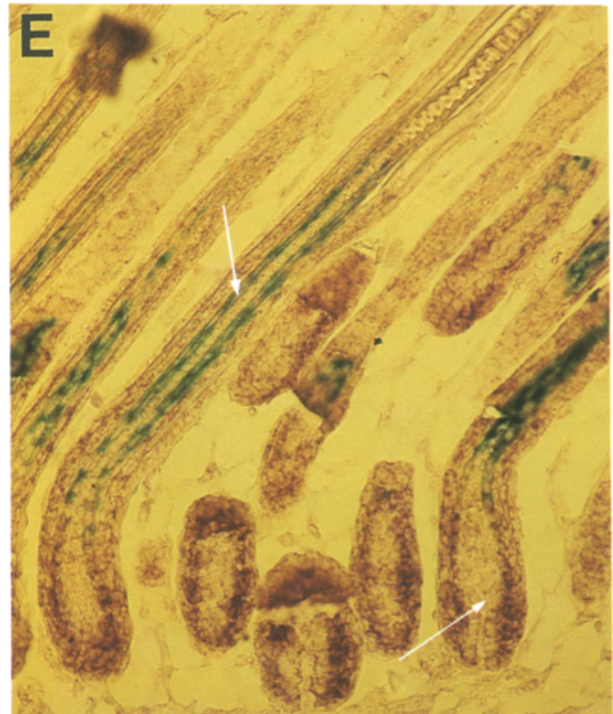
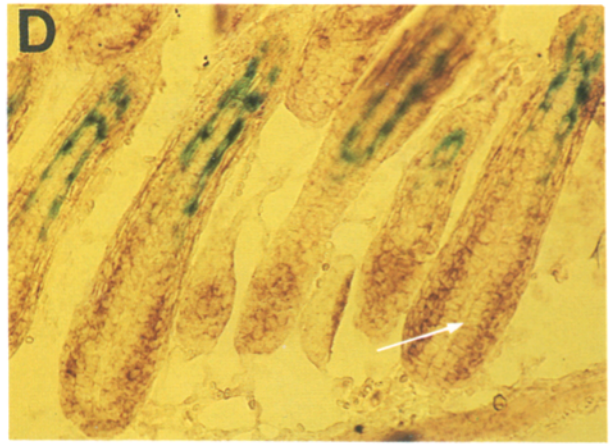
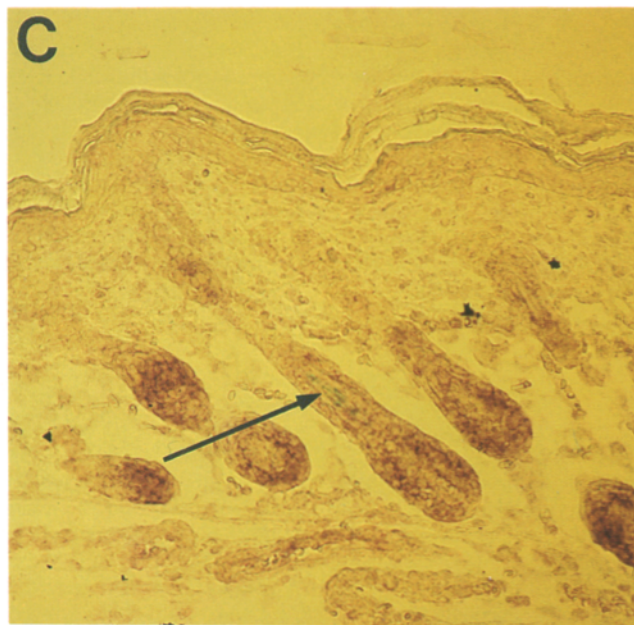
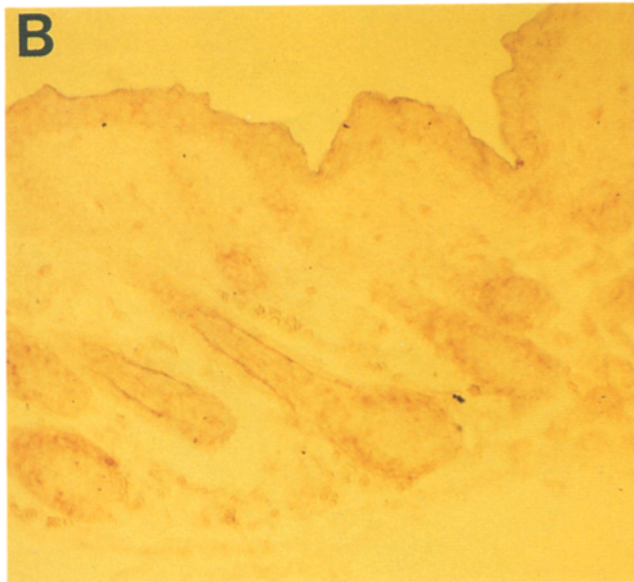
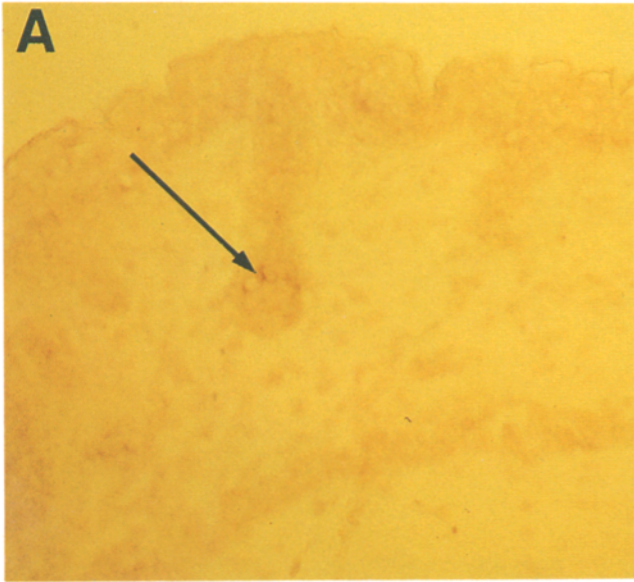


Figure 4. Id expression in whisker follicle. Whole-mount ISH was performed as described in Fig. 3. Day 11 pc (A), day 12 pc (B), and day 13 pc (C) embryos were hybridized with antisense Id RNA probe and stained. Signal is detected in limb buds, especially in marginal zones in day 11 and 12 pc embryos and in the necrotic zone of day 13 pc embryos. Arrows mark sites of Id expression in the maxillary component of the first pharyngeal arch of day 11 and day 12 embryos. The Id signal resolves into rings around developing whiskers in day 13 embryos (note the webbed limb and compare to Fig. 3 C. On day 14 dewebbing has started, 3D). Abbreviations are the same as in Fig. 3.

(*psm*). (B) Day 12 pc embryo, presented from a dorso-lateral viewpoint, showing signal in spinal cord and dorsal root ganglia, but not in pharyngeal arches. (C) Day 13 pc showing signal in presomitic mesoderm but not in whiskers, even though the developing follicles are clearly visible (*arrows*). At day 14 pc, mNotch signal is detectable in the center of each follicle (D, *arrow*). For orientation, forelimb bud is labeled (*flb*).



orbital whiskers. Since the sensitivity of whole-mount ISH may not be high enough to detect low level expression in isolated cells, we cannot rule out the possibility that low-level expression of mNotch is present in skin at these stages or that a small number of individual cells even express mNotch at high levels. Other Notch homologs may be expressed in non-overlapping domains during skin development.

Given the anticipated relationship of hair pattern to *D. melanogaster* bristle formation, the lack of a clear signal from skin at these stages and at this level of detection was unexpected. To verify that other probes generated from moderately expressed mRNAs expressed in skin during early morphogenetic events could be detected, we analyzed the expression pattern of Id, a member of the bHLH family of transcription regulators (Benezra et al., 1990; Weintraub et al., 1991; Wang et al., 1992). In early embryonic ages, expression was abundant throughout the embryo (data not shown). At day 11 pc (Fig. 4 A) a prominent signal could be seen in the CNS, developing limb bud (fb), the pharyngeal arches (pha), the spinal cord (sc) and dorsal root ganglia (drg). No signal was detected in presomitic mesoderm (compare with Fig. 3). The signal in the first pharyngeal arch included rings of more intense staining (Fig. 4 A, arrow). At day 12 pc the signal at the maxillary component of the first pharyngeal arch resolved into four rings in two rows (Fig. 4 B, arrows; compare with lack of pharyngeal arch staining in Fig. 3 B), thus, marking the first stages of overt whisker development at the site and time described by histological observations (Kanno et al., 1991; Kopan and Fuchs, 1989; Hardy, 1992). Day 13 pc embryos had rings of Id expression in five rows in the mesenchyme surrounding the developing whisker. Rings of Id expression mark the site of paraorbital whiskers as well (Fig. 4 C, arrows). Id expression in whiskers of day 13 embryos preceded detection of mNotch expression by a day. As the whiskers were the first hair follicles to develop, Id expression not only provided a visual marker of whisker development but also stood in contrast to the lack of detectable signal with mNotch probes in the pharyngeal arches at day 11 pc, day 12 pc, and day 13 pc (Fig. 3, A-C). Since all probes used for ISH were hydrolyzed to an average size of 100–110 nucleotides, differential penetration by probes could not account for these observations. Thus, mNotch expression detected in the center of whisker follicles at day 14 pc (Fig. 3 D) lagged behind whisker induction and the early stages of whisker morphogenesis (see Hardy, 1992 and references therein for stages of follicle development).

Spatiotemporal Expression Patterns of mNotch throughout Hair Development and in Individual Hair Follicle Cells

To investigate the spatiotemporal pattern of mNotch expres-

sion during the differentiation of individual cells in the hair follicle, we conducted a series of double-labeled ISH. Given the identical patterns observed with all probes at the whole-mount ISH level, and since no evidence for skin expression of the rat Notch II gene were reported (Weinmaster et al., 1992) the data in the following sections were obtained with pMotch as the probe for mNotch and with pKSV15 to detect cortex-specific keratin expression as a differentiation marker (Kopan and Fuchs, 1989; and see Materials and Methods for detail). Cross sections of back skin from day 14 pc, day 17 pc, day 18 pc, newborn, and samples covering every other day from birth to day 34 pp were analyzed. mNotch mRNA was first detected at day 14 pc in cross sections of whiskers (data not shown; same as in hair, see below). By day 18 pc, mNotch was detectable in a few cells located at the bulb region of follicles at the stage in hair development when inner root sheath cells first appear (stage 4 follicle according to Hardy 1992; Fig. 5 A, two cells by the arrow). At birth the follicle had detectable mNotch mRNA expression in the hair matrix (Fig. 5 B). At day 1 pp mNotch mRNA expression was elevated in the matrix, and for the first time the keratin signal was visible (Fig. 5 C, arrow; see also Kopan and Fuchs, 1989). Examination of sections from day 4 pp (Fig. 5 D) and day 10 pp (Fig. 5 E) revealed strong expression of mNotch mRNA in the matrix (confirming the expression pattern observed in the hair follicle of this age by Weinmaster et al., 1991). The specific cell types expressing mNotch were the precursor cells of cortex, the cuticle and inner root sheath cells (see Fig. 1). This expression pattern continued throughout the growth phase of the hair cycle. In the outer root sheath, in matrix cells adjacent to dermal papillae, and in cells contributing to the hair medulla, expression of mNotch mRNA was not detectable (Fig. 5, D–F, white arrow, and F, hollow arrow). In Huxley's layer cells, cuticle, and hair cuticle, mNotch mRNA was only visible in the lower portion of the matrix (Fig. 5 F, black arrow). Finally, a close examination of hair follicles revealed that mNotch mRNA was present in terminally differentiating cells located in Henle's layer and was coinciding with keratin expression in terminally differentiating cortex cells (Fig. 1, and Fig. 5, E and F).

mNotch Maintains the Same Spatiotemporal Expression Patterns during the Second Hair Cycle: No Expression Is Detectable in Stem Cells

The mammalian hair cycle is unique in that it regenerates hair follicles throughout adult life. The stem cells contributing to cycling follicles are thought to be situated in the bulge, the attachment site of the arractor pili muscle, located in the upper third of the follicle (see Costarelis et al., 1990 for detail). As the first growth phase comes to an end, a follicle degradation phase (catagen) destroys the lower two thirds of

Figure 5. Expression of mNotch in hair follicle during development. Mouse skin sections were hybridized simultaneously with a biotin 11 UTP antisense wool type I keratin RNA probe and a digoxigenin-labeled antisense mNotch RNA probe. Detection of digoxigenin-labeled probe was done with alkaline-phosphatase-conjugated antibodies (brown). Biotinylated probe was detected by streptavidin- β -galactosidase conjugate (blue). By day 18 pc, mNotch is detectable in a few cells located at the bulb region of follicles (A, arrow). At birth, mNotch mRNA is present in the hair matrix (B). In 1-d-old mice, mNotch mRNA expression is elevated in the matrix, and for the first time the Keratin signal is visible (C, arrow). 4-d-old mice (D) and 10-d-old mice (E) have strong expression of mNotch mRNA in the matrix. The specific cell types expressing mNotch are the precursor cells of cortex, cuticle, and inner root sheath cells. In outer root sheath, matrix cells adjacent to dermal papillae, and in cells contributing to the hair medulla, expression of mNotch mRNA is not detectable (D–F, white arrows and F, hollow arrow). In Huxley's layer cells, cuticle and hair cuticle mNotch mRNA is only visible in the lower portion of the matrix (D and E; in F, black arrow at the level of three cells that are differentiating into Huxley's layer and the two cuticle cell layers). mNotch mRNA is present in terminally differentiating cells located in Henle's layer and is overlapping keratin expression in terminally differentiating cortex cells (F, arrow heads).

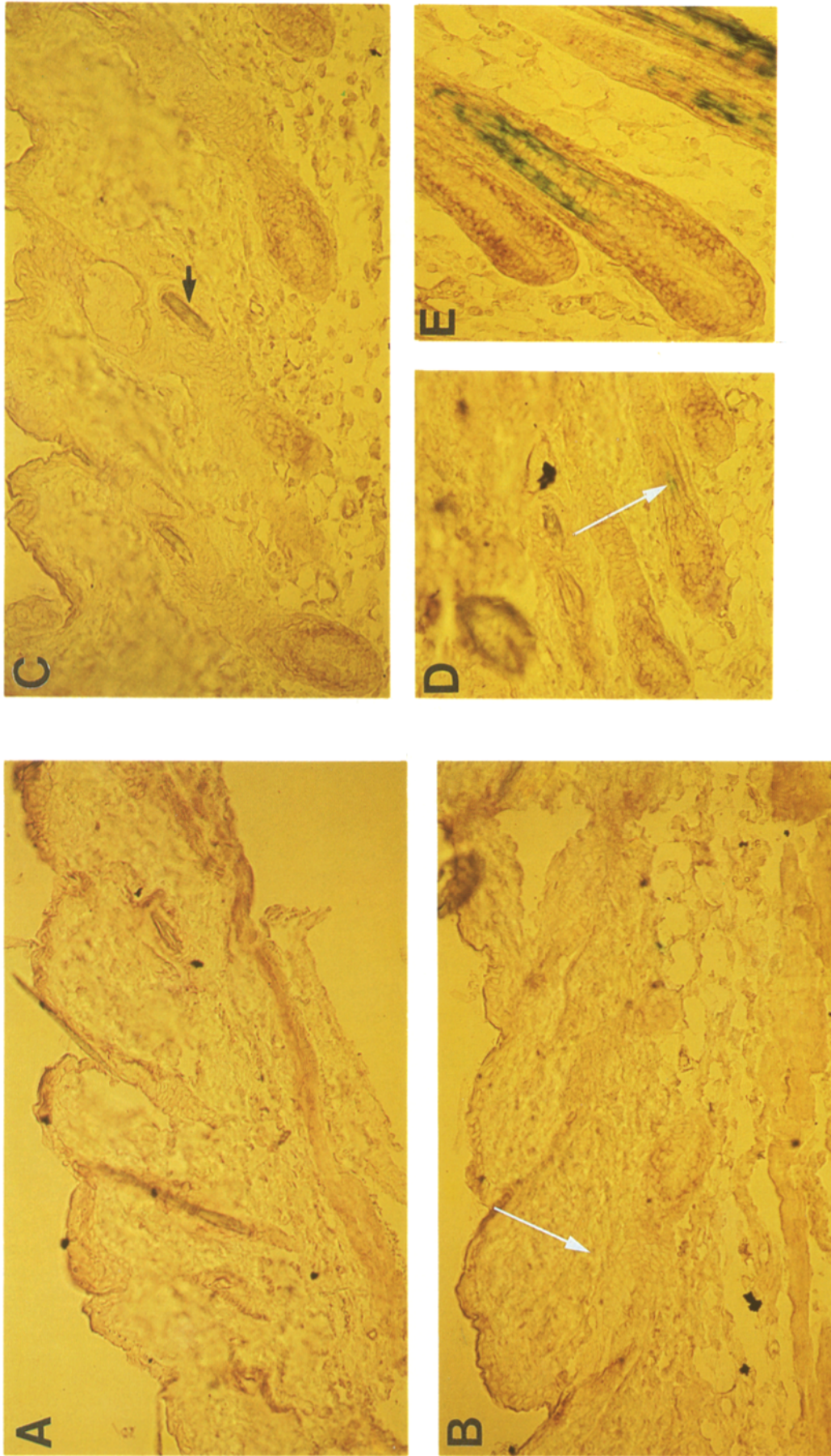


Figure 6. Expression of mNotch in hair follicle during the second hair cycle. Mouse skin sections were hybridized simultaneously with a biotin 11 UTP antisense wool type I keratin RNA probe and a digoxigenin-labeled antisense mNotch RNA probe. Detection of digoxigenin-labeled probe was done with alkaline-phosphatase conjugated antibodies (brown). Biotinylated probe was detected by streptavidin- β -galactosidase conjugate (blue). Resting skin from day 22 pp shows no signal above background level (A). No mNotch expression is detected in the bulge at the start of the new cycle at day 24 pp (B, bulge located under arrow). At day 26 pp the first detectable mNotch mRNA is located in matrix in the equivalent of the hair follicle (C). At day 29 pp cortex differentiation is evident (blue, arrow) trailing behind morphogenesis as it did during embryonic development of the follicle (D). At day 33 pp, hybridization patterns are identical with first cycle follicles (E, compare with Fig. 5). In most sections, notice the club hair left from the first cycle (arrow, C).

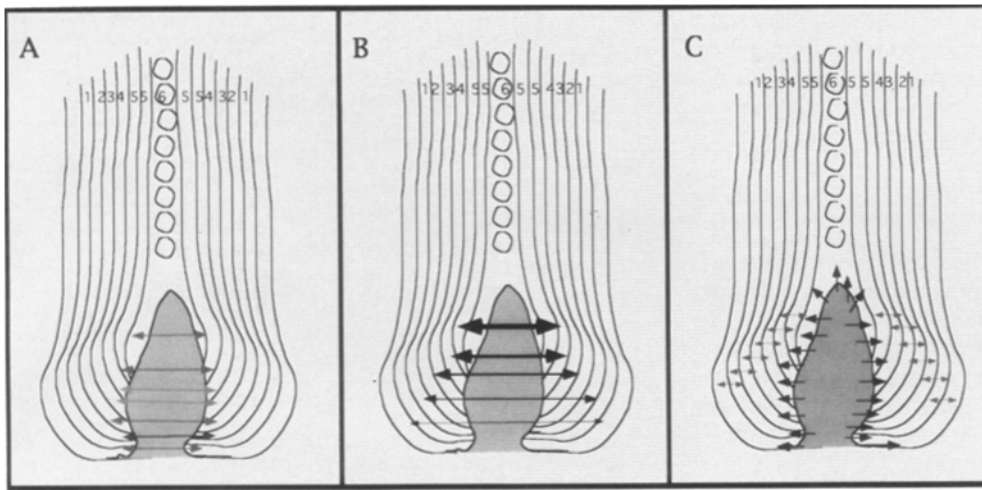


Figure 7. Generation of different cell types in the hair follicles. (A) Dermal papilla delivers six specific signals to generate lineage-restricted stem cells in the bulb. These cells give rise to six different cell layers. (B) Dermal papilla generates a single morphogen field that is interpreted by matrix cells giving rise to six different cell layers. (C) A dermal papilla signal(s) is received by matrix cells. Matrix cells communicate, select specific fates, and give rise to six different cell layers.

the follicle. Dermal papillae cells are then in close proximity to the bulge, and after a rest phase (telogen) a new follicle is formed (Costarelis et al., 1990). In these studies we have established that high levels of mNotch are expressed in the skin after hair and whisker morphogenesis has begun. Is mNotch expressed in the hair follicle during the second cycle?

Since the first several hair cycles in mice are synchronous, we were easily able to test the temporal and spatial distribution of mNotch mRNA throughout the cycles. In the second cycle, the expression profile of mNotch paralleled the pattern observed during embryonic follicle formation in the first cycle. There was no signal above background in skin during the follicle degradation phase (data not shown) or in resting skin from day 17 pp to day 22 pp (Fig. 6 A, day 22 pp). Interestingly, no mNotch expression was detected in the bulge at the start of the new cycle (day 24 pp; Fig. 6 B, arrow; and data not shown). At day 26 pp the first detectable mNotch mRNA was located in matrix in the stage 4 follicle (Fig. 6 C). At day 29 pp, cortex differentiation was evident, trailing behind morphogenesis as it did during embryonic development of the follicle (Fig. 6 D, arrow; and see Fig. 5) (Kopan and Fuchs, 1989). At day 33 pp, the pattern of hybridization for both probes was indistinguishable from that of a day 10 pp follicle (compare Fig. 6 E to Fig. 5, D–F).

Discussion

Stem Cell Maintenance and Early Stages during Epithelial–Mesenchymal Interactions in Skin Are Not Correlated with High Level of mNotch Expression

Within the embryonic skin, localized, reciprocal epithelial–mesenchymal interactions lead to the production of individual hair follicles with specific morphological features. The distribution of hair (or feather) follicles produces patterns in vertebrate skin (Sengel, 1976). Presumably, the initiation of this complex process begins with a localized signal coming from either embryonic ectoderm or the mesoderm component of skin. Resolution of a sheath of cells into signal- vs. nonproducing cells (Davidson, 1983; Linsenmayer, 1972) superficially resembles bristle formation in *D. melanogaster* (resolving a sheath of cells into epidermis

vs. neurons). Though one might have expected mNotch to be involved in this process based on the *D. melanogaster* paradigm, our ISH failed to detect high level expression of mNotch in ectoderm or mesoderm between the time of somite segmentation and developing hair follicle formation as assayed in whole mounts (Fig. 5) and in cross sections (not shown). However, the ISH technique did detect Id mRNA in the anticipated spatiotemporal context. Although we can not rule out that some low level of mNotch mRNA is below the sensitivity of the assay, high level Notch expression is known to be associated with function in *D. melanogaster* (Fehon et al., 1991; Ruohola et al., 1991). Moreover, other Notch homologs may be expressed in skin during these stages.

Stem cells populate several sites within the skin: the basal layer of the epidermis, the rete ridges of the palm, and the bulge of the hair follicle (Costarelis et al., 1990). At no time during skin development, growth, or the hair cycle were high levels of mNotch mRNA detected in the basal epidermal cells or in the bulge cells. mNotch is absent from the cell layer adjacent to dermal papillae, previously thought to be the site of hair stem cells and now considered to be a transiently dividing, multipotent cell population (Costarelis et al., 1990). These cells display high mitotic activity (Tezuka et al., 1990 and references therein) and are thought to give rise to all cell types within the follicle (Sengel, 1976). Previous observations of Notch expression in hair follicles (Weinmaster et al., 1991; Franco Del Amo et al., 1992) did not allow the complete identification of the cell types involved in formation of the follicle or their mitotic status. The absence of detectable mNotch mRNA from skin stem cells at any time in the hair cycle, its absence from the most actively dividing cells within the matrix, and its abundant presence in growing cells prior to and coincident with overt signs of differentiation, argues against a role in maintenance of a less differentiated state in stem cells or involvement in proliferation. Studies of follicle induction by dermal papilla have established the short range nature of the inductive signal: if direct contact between epidermal cells and dermal papilla is not maintained, follicular differentiation is not observed (Oliver and Jahoda, 1988; Jahoda, 1992). mNotch is therefore less likely to participate directly in epithelial–mesenchymal signaling as it is expressed in cells that do not contact the dermal papilla. Though expression of mNotch correlates

with the proposed role in maintaining a differentiated state, we feel that the pattern of mNotch expression reported here also supports a role for mNotch in binary cell fate decisions (see below).

The Acquisition of Cellular Identities within Hair Follicle

Sengel (Sengel, 1976) has suggested that "according to precisely where they (matrix cells) are within the hair bulb, they follow six or seven different differentiative pathways" (Fig. 1). This observation could be the result of different molecular mechanisms: either cells in the matrix are all descendant of seven different lineage restricted stem cells, positioned around the dermal papillae (Fig. 7, A and B) or cell-cell interactions lead to cell fate choice (Fig. 7 C).

The size of a hair follicle is controlled by the number of cells in the dermal papilla (Ibrahim and Wright, 1982; Johoda, 1992). However, it is not clear if generation of distinct cell types in hair follicles is also controlled by the dermal papilla as it is in the avian feather (Sengel, 1976). Three possible mechanisms can be considered for the generation of cell diversity in hair follicles. The first model describes dermal papillae as producing six different, short range signals leading to formation of lineage restricted proliferating cells in the hair matrix (Fig. 7 A). In a second, similar model (Fig. 7 B), dermal papillae cells produce one signal that diffuses to form a gradient of concentrations which is differentially read at seven different levels (Fig. 7 B). The third model, in its extreme, requires only a single signal, followed by a fate selection process between cells in the epithelial component of the follicle (Fig. 7 C). As mentioned above, localization of mNotch mRNA in the follicle cells does not correlate well with a role for mNotch as a receptor for mesenchymal signals of the nature presented in Fig. 7 (A and B). Since neighboring cells within the hair follicle select different differentiation pathways, binary cell fate choices may occur in a precursor matrix cell population in a manner similar to that seen in invertebrates. If mNotch plays a role in cell fate choice, the expression patterns of mNotch during follicle development are consistent with the model presented in Fig. 7 C. We are currently performing experiments which allow direct testing of these models.

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