DEVELOPMENTAL BIOLOGY **204,** 420–431 (1998)
ARTICLE NO. DB989092

Delta–Notch Signaling in Odontogenesis: Correlation with Cytodifferentiation and Evidence for Feedback Regulation

Thimios A. Mitsiadis,*,†,[1] Estelle Hirsinger,† Urban Lendahl,‡ and Christo Goridis†

**Faculté d'Odontologie, Université de la Méditerranée, IMEB EA 2198, 13916 Marseille Cedex 20, France; †Laboratoire de Génétique et Physiologie du Développement, IBDM, CNRS/INSERM/Université de la Méditerranée/AP de Marseille, Campus de Luminy Case 907, 13288 Marseille Cedex 9, France; and ‡Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, S-171 77 Stockholm, Sweden*

Recent data suggest that dental cells utilize the evolutonarily conserved Notch-mediated intercellular signaling pathway to regulate their fates. Here we report on the expression and regulation of Delta1, a transmembrane ligand of the Notch receptors, during mouse odontogenesis. *Delta1* is weakly expressed in dental epithelium during tooth initiation and morphogenesis, but during cytodifferentiation, expression is upregulated in the epithelium-derived ameloblasts and the mesenchyme-derived odontoblasts. The expression pattern of *Delta1* in ameloblasts and odontoblasts is complementary to *Notch1*, *Notch2*, and *Notch3* expression in adjacent epithelial and mesenchymal cells. Notch1 and Notch2 are upregulated in explants of dental mesenchyme adjacent to implanted cells expressing *Delta1*, suggesting that feedback regulation by Delta–Notch signaling ensures the spatial segregation of Notch receptors and ligands. TGFβ1 and BMPs induce *Delta1* expression in dental mesenchyme explants at the stage at which *Delta1* is upregulated *in vivo*, but not at earlier stages. In contrast to the Notch family receptors and their ligand Jagged1, expression of *Delta1* in the tooth germ is not affected by epithelial–mesenchymal interactions, showing that the Notch receptors and their two ligands Jagged1 and Delta1 are subject to different regulations.

Key Words: **neurogenic genes; Notch; Delta; TGFβ; tooth; odontoblast.**

INTRODUCTION

Notch-like transmembrane receptors and cell-bound ligands of the Delta/Serrate type mediate phylogenetically conserved cell communication processes which enable neighboring cells to adopt different fates (Artavanis-Tsakonas *et al.*, 1991, 1995 ; Nye and Kopan, 1995; Simpson, 1995; Weinmaster, 1997). Expression of Delta or Serrate on a cell among a group of initially equivalent cells allows this cell to acquire a given fate and, at the same time, instructs the surrounding cells, via activation of their Notch receptors, to adopt a different fate or to remain undifferentiated (a mechanism referred to as lateral specification or inhibition, respectively). Although Delta and Serrate can substitute for each other in some functions (Gu *et al.*, 1995; Sun and Artavanis-Tsakonas, 1996), they are not functionally equivalent, since Delta mutations can be only partially rescued by ectopic expression of Serrate (Gu *et al.*, 1995) and since dominant-negative forms of Delta and Serrate expressed in identical domains of the *Drosophila* eye do not produce the same phenotypes (Sun and Artavanis-Tsakonas, 1996). By analogy with *Drosophila*, Notch signaling in vertebrates is believed to control both commitment of cells to differentiate as well as choices between alternative differentiation pathways. Notch family members have been implicated in the development of a broad spectrum of vertebrate tissues and organs, such as the neural tube, somites, eyes, hairs, and scales (Kopan and Weintraub, 1993; Kopan *et al.*, 1994; Conlon *et al.*, 1995; Henrique *et al.*, 1995, 1997; de la Pompa *et al.*, 1997; Dorsky *et al.*, 1997; Crowe and Niswander, 1998).

[1] To whom correspondence should be addressed. E-mail: mitsiadi@ibdm.univ-mrs.fr.

0012-1606/98 $25.00

Recent work suggests that Notch receptors and ligands may be important also during odontogenesis (Mitsiadis *et al.*, 1995, 1997). Teeth develop as a result of sequential and reciprocal interactions between neural crest-derived mesenchyme and the oral ectoderm. Such tissue interactions gradually transform the initially homogeneous epithelial and mesenchymal components of the tooth primordium into complex structures. These interactions can be studied in dissected pieces of dental epithelium and mesenchyme cultured as recombinants, while gene regulation by extracellular factors can be analyzed using beads preloaded with signaling molecules and implanted into dental explants (Thesleff *et al.*, 1995). Expression of both Notch receptors (Mitsiadis *et al.*, 1995) and the Jagged1 (Jag1) ligand (Mitsiadis *et al.*, 1997) in the epithelial components of the molar tooth primordium prefigures the subdivision of the epithelium into ameloblastic (capable of enamel-matrix synthesis) and nonameloblastic regions already at the initiation stage. Although these results suggest a role for the Notch receptors and Jag1 in the control of early odontogenesis, it remains uncertain if the diversity of dental cell types is dependent on lateral specification mediated by Notch signaling. Four cell layers form the dental epithelial component during late odontogenesis: the inner enamel epithelium (giving rise to the ameloblasts), stratum intermedium, stellate reticulum, and outer enamel epithelium. The dental mesenchyme is also composed of different cell types such as odontoblasts, cells of the subodontoblastic layer, dental papilla cells, and cells of the dental follicle. Ameloblasts and odontoblasts are highly differentiated cells, responsible for the formation of the hard tissues of the teeth (the enamel and the dentin, respectively), while cells of the dental follicle contribute to the formation of the periodontium (which links the teeth to the alveolar bone). However, the biological functions of the other dental cell types are not well understood. Here we extend the analysis on the Notch-mediated signaling during odontogenesis to the mouse *Delta1* (*Dll1*) gene. Our data show that *Dll1* expression correlates with ameloblast and odontoblast differentiation and is regulated by BMPs[2] and TGFβ1 and suggest that Dll1 upregulates Notch1 and 2 expression in neighboring cells.

[2] Abbreviations used: BMP, bone morphogenic protein; TGF, transforming growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin

MATERIALS AND METHODS

Animals and Tissue Preparation

Swiss and F_1 (CBA × C57/BL) or (CBA × NMRI) mice were used at embryonic stages (embryonic day 10.5 to embryonic day 18.5; E10.5–E18.5). The age of the mouse embryos was determined according to the appearance of the vaginal plug (day 0.5) and confirmed by morphological criteria. The embryos were surgically removed in Dulbecco's phosphate-buffered saline (PBS). Dissected heads from mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA).

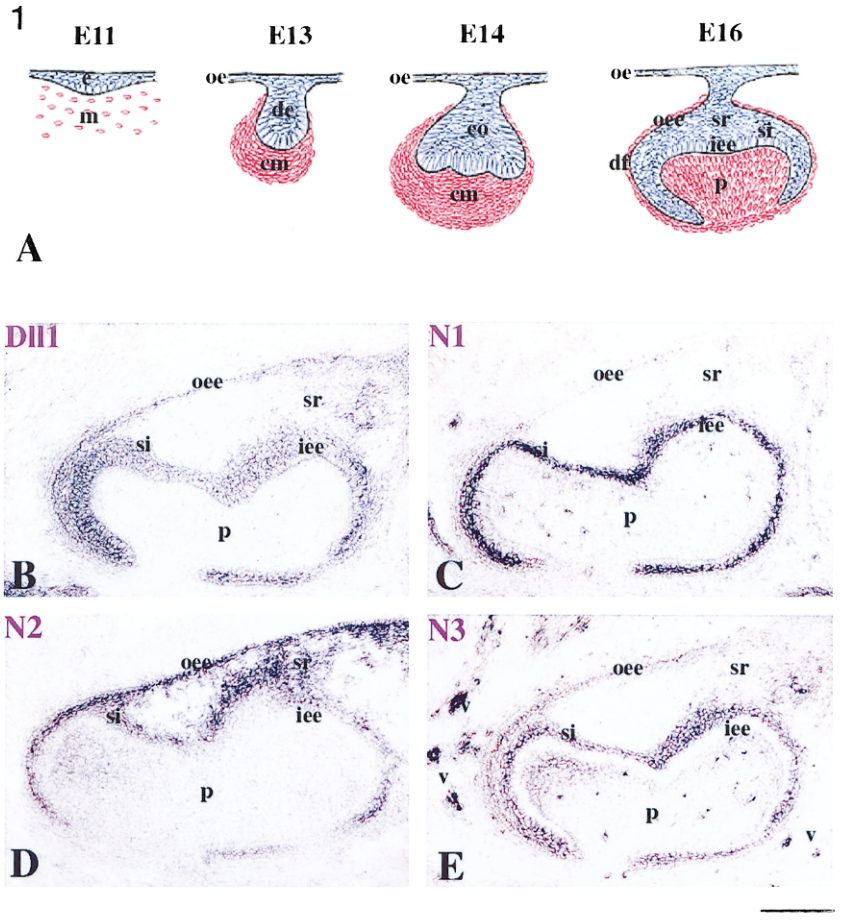
Probes, Antibodies, in Situ Hybridization, and Immunohistochemistry

For *in situ* hybridization studies, single-stranded [^{35}S]UTP- and digoxigenin-labeled antisense riboprobes for mouse *Dll1* (Bettinghausen *et al.*, 1995), chick *Dll1* (*cDll1;* Henrique *et al.*, 1997), chick *MyoD* (Pourquié *et al.*, 1996), and *Notch1*, *Notch2*, and *Notch3* (Mitsiadis *et al.*, 1995) were synthesized as described (Mitsiadis *et al.*, 1997). For immunohistochemistry, rabbit polyclonal antibodies against the extracellular domain of the mouse Notch1, Notch2, and Notch3 proteins (Mitsiadis *et al.*, 1995 and Feli *et al.*, submitted for publication) were used. Whole-mount *in situ* hybridization and immunohistochemistry on explants and *in situ* hybridization on cryosections and on paraffin sections were performed as previously described (Mitsiadis *et al.*, 1995, 1997).

Coculture of Dental Explants with Quail QT6 Cells

Quail QT6 cells (Pourquié *et al.*, 1996) and QT6 cells infected with *cDll1* were a gift from Dr. Olivier Pourquié (IBDM, Marseille, France). Dental mesenchyme was isolated from E16.5–E17.5 lower molar tooth germs after a 10-min incubation in 3% pancreatin. Clumps of QT6 cells were either placed on top or implanted into dental mesenchyme explants and then cocultured for 24 h. After culture, explants were fixed in 4% PFA and then processed for whole-mount *in situ* hybridization and immunohistochemistry.

FIG. 1. Comparison between *Delta1 (Dll1), Notch1 (N1), Notch2 (N2),* and *Notch3* (*N3*) expression in a mouse molar at the early bell stage. Photomicrographs of *in situ* hybridizations on cryosections using digoxigenin-labeled probes are shown. (A) Schematic representation of the different stages of molar development: placode (E11), bud (E13), cap (E14), and early bell (E16) stages. Dental epithelium in blue, dental mesenchyme in red. (B) *Dll1* mRNA expression in the inner enamel epithelium (iee), stratum intermedium (si), stellate reticulum (sr), and outer enamel epithelium (oee). Note the absence of *Dll1* expression in the dental papilla (p). (C) Strong *N1* mRNA expression is found in cells of the stratum intermedium. (D) *N2* transcripts are found in the stellate reticulum, stratum intermedium, and outer enamel epithelium and at low levels in the cuspal area of the dental papilla. (E) *N3* transcripts are localized in the stratum intermedium, the cuspal area of the dental papilla, and vascular structures (v). Some transcripts are also detected in cells of the outer enamel epithelium. Abbreviations: df, dental follicle; oe, oral epithelium; cm, condensed mesenchyme; eo, enamel organ; m, mesenchyme; e, epithelium; de, dental epithelium; oee, outer enamel epithelium; iee, inner enamel epithelium; sr, stellate reticulum; si, stratum intermedium; p, dental papilla; v, vessels. Size bar, 100 μm.



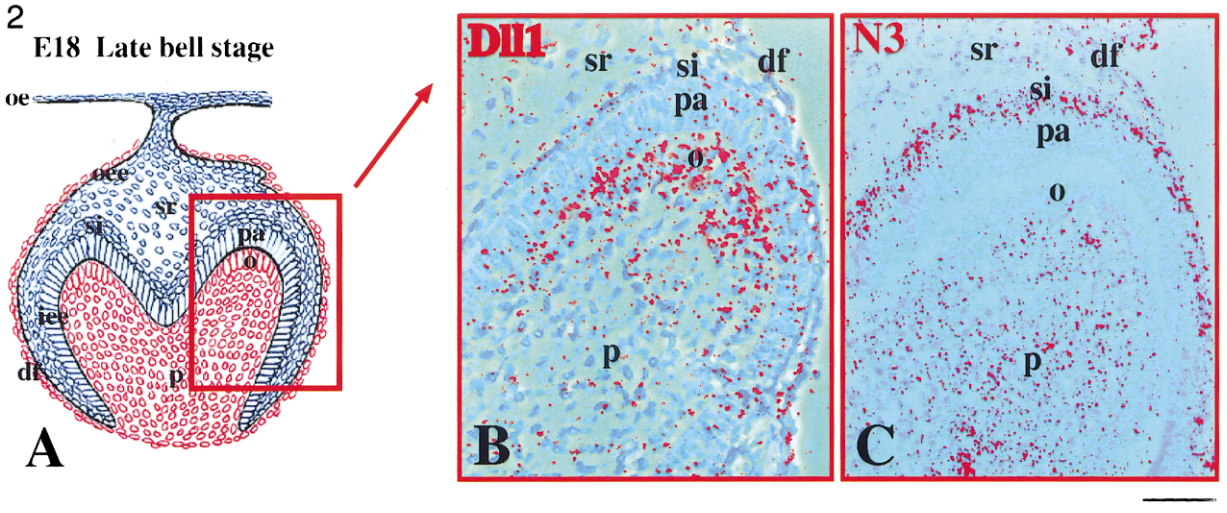
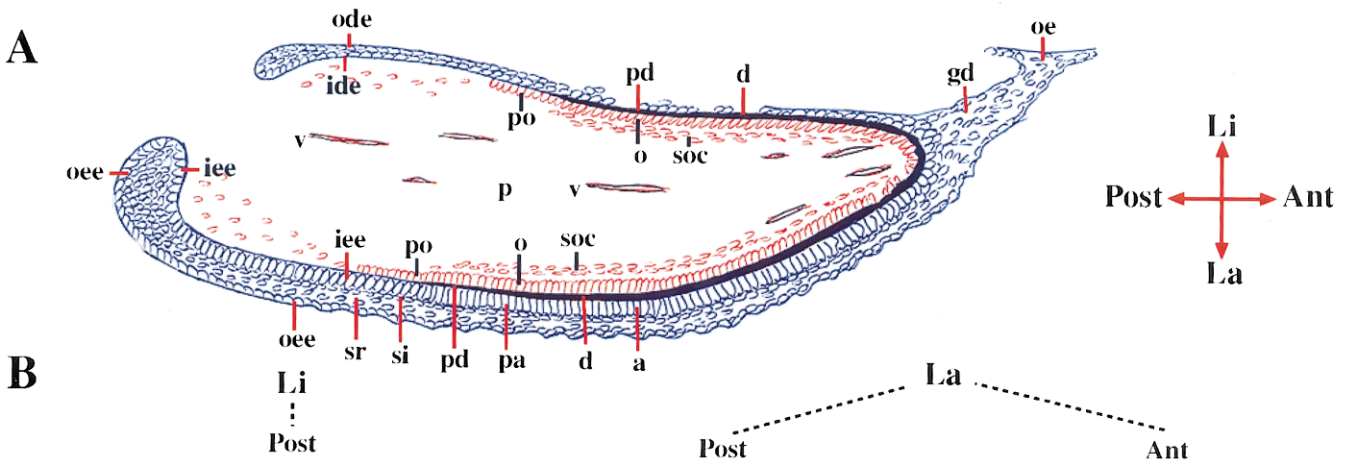
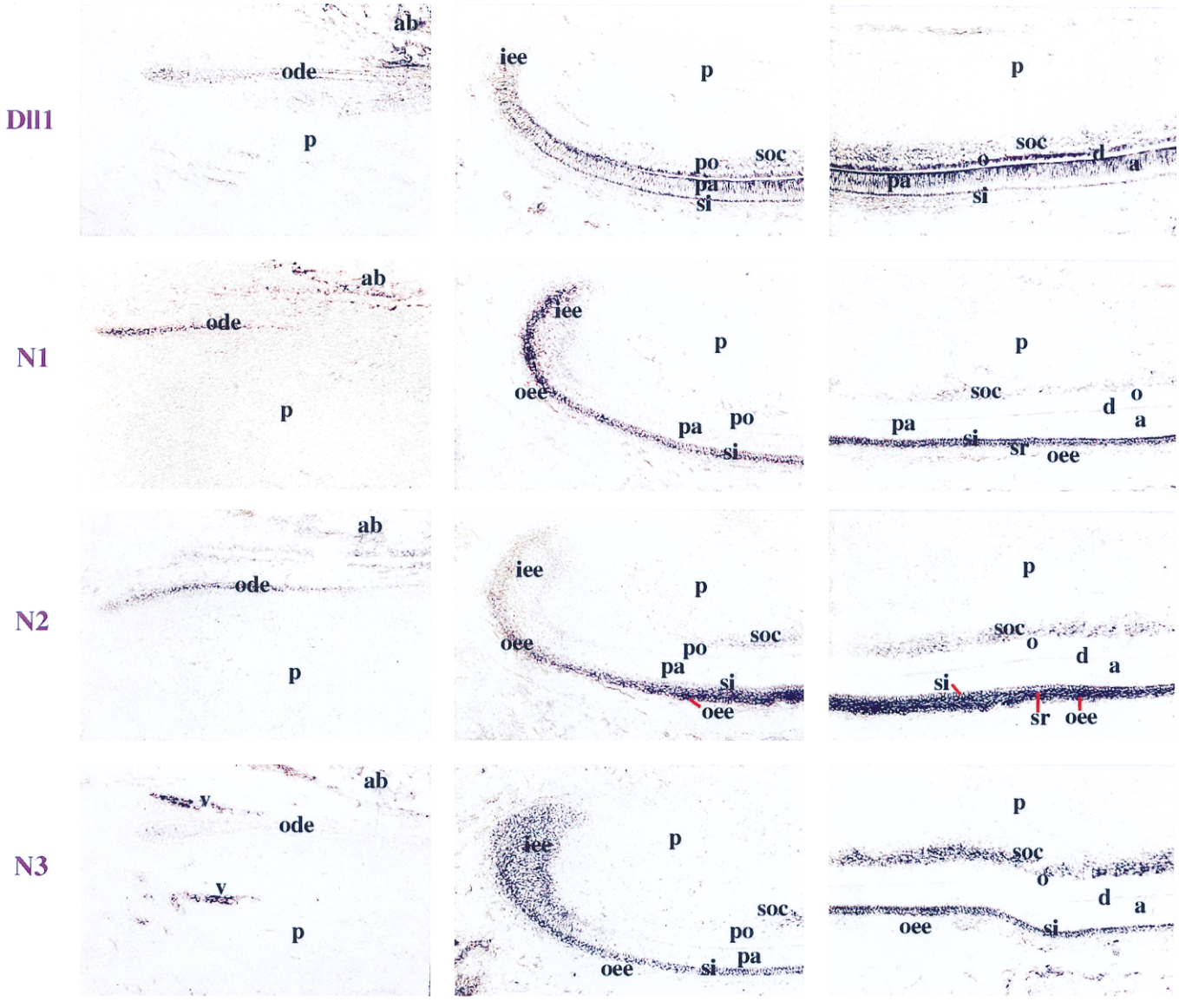


FIG. 2. Comparison between *Dll1* and *N3* expression during odontoblast differentiation of a mouse molar. Photomicrographs of *in situ* hybridizations on paraffin sections with ^{35}S-labeled probes are shown. The *in situ* signal (dark field) is shown in red and the morphology of the tissue (bright field) is shown in blue. (A) Schematic representation of a molar at the late bell stage (E18). Dental epithelium in blue, dental mesenchyme in red. (B and C) Framed area from (A). (B) Intense *Dll1* expression is observed in differentiating odontoblasts (o). (C) *N3* transcripts are found in the stratum intermedium (si) and the dental papilla (p), but are absent from preameloblasts (pa) and odontoblasts. Other abbreviations as in Fig. 1. Size bar, 50 μm.





Tissue Recombination Experiments

For tissue recombination (epithelium–mesenchyme) and bead implantation experiments, lower molar tooth germs from E12.5 to E16.5 mouse embryos were used. After dissection from the rest of the mandible, the tooth germs were incubated for 5 min in 2.25% trypsin and 0.75% pancreatin on ice, and the epithelia were mechanically separated from mesenchyme in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum (Gibco). The isolated epithelia were placed in contact with isolated mesenchyme and cultured for 24 h on a polycarbonate membrane (Nuclepore Corp.) as previously reported (Mitsiadis *et al.,* 1995, 1997). After culture, the recombinants were fixed for 2 h in 4% PFA and then treated as whole mounts.

Recombinant Proteins and Treatment of the Beads

Recombinant FGF4 (British Biotechnology Products), BMP2, and BMP4 (a gift from Dr. E. Wang, Genetics Institute, Cambridge, MA) were used to preload beads (1 μl of a solution of 100–250 μg/ml per 10 beads). As a control, we used beads preloaded with 0.1% BSA in PBS. Beads were transferred on top of dental explants, and after 24 h of culture the explants were fixed in 4% PFA (for details see Mitsiadis *et al.,* 1995, 1997).

RESULTS

Expression of Dll1 during Odontogenesis: Comparison with Notch1, Notch2, and Notch3 Expression

Sections from different stages of molar tooth development (summarized in Figs. 1A and 2A) were analyzed by both radioactive and nonradioactive (digoxigenin) *in situ* hybridization in mouse embryos. During the stages of tooth initiation (E11.5) and early tooth morphogenesis (bud and cap stages, E12.5–15.5), *Dll1* mRNA expression is observed at very low levels in dental epithelium but not in dental mesenchyme (data not shown). During the early bell stage (E16.5), *Dll1* expression increases in the enamel organ, where it is now strongly expressed in the inner enamel epithelium and the stratum intermedium and more weakly in the stellate reticulum and the outer enamel epithelium (Fig. 1B). We compared the expression patterns of the *Notch* genes with that observed for *Dll1* on serial sections. In the epithelial derivatives, *Notch1* expression is restricted to cells of the stratum intermedium (Fig. 1C), *Notch2* transcripts are detected in the stratum intermedium, the stellate reticulum, and the outer enamel epithelium (Fig. 1D), and *Notch3* is expressed in cells of the stratum intermedium and the outer enamel epithelium (Fig. 1E). In contrast to *Notch1* and *Notch3, Notch2* is only weakly expressed in the cervical loop region (i.e., the most ventral part of the molar). In the dental papilla, *Notch1* transcripts are detected in vascular structures, *Notch2* is weakly expressed in the cuspal mesenchyme, and *Notch3* mRNA is found in mesenchymal cells of the cusps and in blood vessels. During cytodifferentiation (late bell stage, E18.5), the terminal division of the mesenchymal preodontoblasts gives rise to two layers of cells with different developmental fates: odontoblasts, which form dentin, and cells of the subodontoblastic layer, of unknown function (Fig. 2A). *Dll1* transcripts are detected for the first time in dental mesenchyme during this stage: a gradient of *Dll1* expression is observed in differentiating odontoblasts, with the strongest signal at the tip of the cusps and progressively lower levels of expression in the developmentally less advanced odontoblasts farther downward, while few transcripts are detected in the subodontoblastic layer (Fig. 2B). By contrast, expression of *Notch1, Notch2,* and *Notch3* during this stage is observed only in mesenchymal cells other than odontoblasts (see Fig. 2C for *Notch3* and Mitsiadis *et al.,* 1995, for the identical expression patterns of *Notch1* and *Notch2*).

The continuously erupting incisor of rodents is morphologically distinct from the molars, forming a cylinder parallel to the long axis of the mandible, with an anterior tip and a widely opened posterior end (Fig. 3A). The incisor offers a unique model of tissue organization involving defined regions of stem cells, differentiating cells, and mature cells and displays distinct morphological organization along both the labial–lingual and the anterior–posterior axes. The dental epithelium at the lingual side of the incisor is composed for the most part of two layers of cells: the outer and inner dental epithelia. An additional third layer of cells (stratum intermedium) can be detected at the posterior end. In contrast, the labial dental epithelium (or enamel organ) is formed by four layers: the inner and outer enamel epithelia, the stellate reticulum, and the stratum interme-

FIG. 3. Comparison between *Dll1, N1, N2,* and *N3* mRNA expression in the lower incisor of an E18.5 mouse embryo. Photomicrographs of *in situ* hybridizations on cryosections with digoxigenin-labeled probes are shown. (A) Schematic representation of a longitudinal section through the lower incisor of an E18.5 mouse embryo. (B) In mesenchymal derivatives, *Dll1* mRNA is expressed in preodontoblasts (po), odontoblasts (o), and cells of the subodontoblastic layer (soc), whereas transcripts for the *N1, N2,* and *N3* genes are only found in cells of the subodontoblastic layer. *N3* mRNA is also detected in vascular structures (v). In the dental epithelium (de) of the labial (La) side, *Dll1* expression is found in cells of the inner enamel epithelium (iee), in preameloblasts (pa), and in ameloblasts (a), while strong *N1* and *N3* signals are detected in the stratum intermedium (si), and *N2* transcripts are found in the stratum intermedium, the stellate reticulum (sr), and the outer enamel epithelium (oee). Lingually (Li), *Dll1* is weakly expressed in the dental epithelium, while a strong signal is seen in the outer dental epithelium (ode) for *N1* and *N2,* but not for *N3.* Other abbreviations: Ant, anterior side; Post, posterior side; p, dental papilla mesenchyme; pd, predentine; d, dentine; ide, inner dental epithelium; ab, alveolar bone; oe, oral epithelium; gd, gubernaculum dentis. Size bar, 200 μm.

dium. The posterior end of the incisor represents a reservoir of cells capable of giving rise to these specific cell populations. There is thus a posterioanterior gradient of cytodifferentiation, with the most differentiated cells being located anteriorly and the most immature ones posteriorly. Only the inner enamel epithelial cells at the labial side differentiate into ameloblasts, whereas the mesenchymal cells underlying both the labial and the lingual epithelia differentiate into odontoblasts.

Sections from E18.5 incisors were analyzed by *in situ* hybridization using digoxigenin-labeled probes. In the epithelial components of the lingual side, *Dll1* mRNA is weakly expressed in the posterior outer and inner dental epithelia (Fig. 3B), while *Notch1* and *Notch2* transcripts are only detected in the very posterior part of the outer dental epithelium (Fig. 3B). At the labial side, the expression patterns of *Dll1* and *Notch1, 2,* and *3* are largely complementary to each other: *Dll1* transcripts are found in cells of the inner enamel epithelium and its derivatives, the preameloblasts and polarizing ameloblasts (Figs. 3B, 4B, and 4D), whereas expression of the three *Notch* genes was strong in the stratum intermedium, but absent from the inner enamel epithelium (except at its posterior end) and from preameloblasts and ameloblasts (Figs. 3B, 4C, and 4E–4G). *Notch2* transcripts are also found in the outer enamel epithelium. At the posterior end, *Notch3* is expressed in the inner enamel epithelium, but becomes progressively restricted to the stratum intermedium as one moves anteriorly, in accordance with the posterioanterior gradient of cytodifferentiation. *Notch1* transcripts are confined to a cell layer in continuity with the stratum intermedium, whereas *Notch2* fades out as one moves posteriorly. Finally, *Dll1* expression is confined to the cells of the inner enamel epithelium destined to become ameloblasts. In the mesenchymal derivatives of E18.5 incisors, the polarizing odontoblasts at both sides express the *Dll1* gene (Figs. 3B, 4A, and 4B). *Dll1* transcripts are also detected in functional odontoblasts synthesizing and secreting the dentin matrix proteins and in cells of the subodontoblastic layer (Figs. 3B and 4D). The three *Notch* genes, by contrast, are only expressed in the cells of the subodontoblastic layer, and not in odontoblasts (Figs. 3B, 4C, and 4E–4G). The weak expression of the *Notch* genes in cells adjacent to still-immature odontoblasts posteriorly contrasts with the strong signal in the subodontoblastic layer as one moves anteriorly.

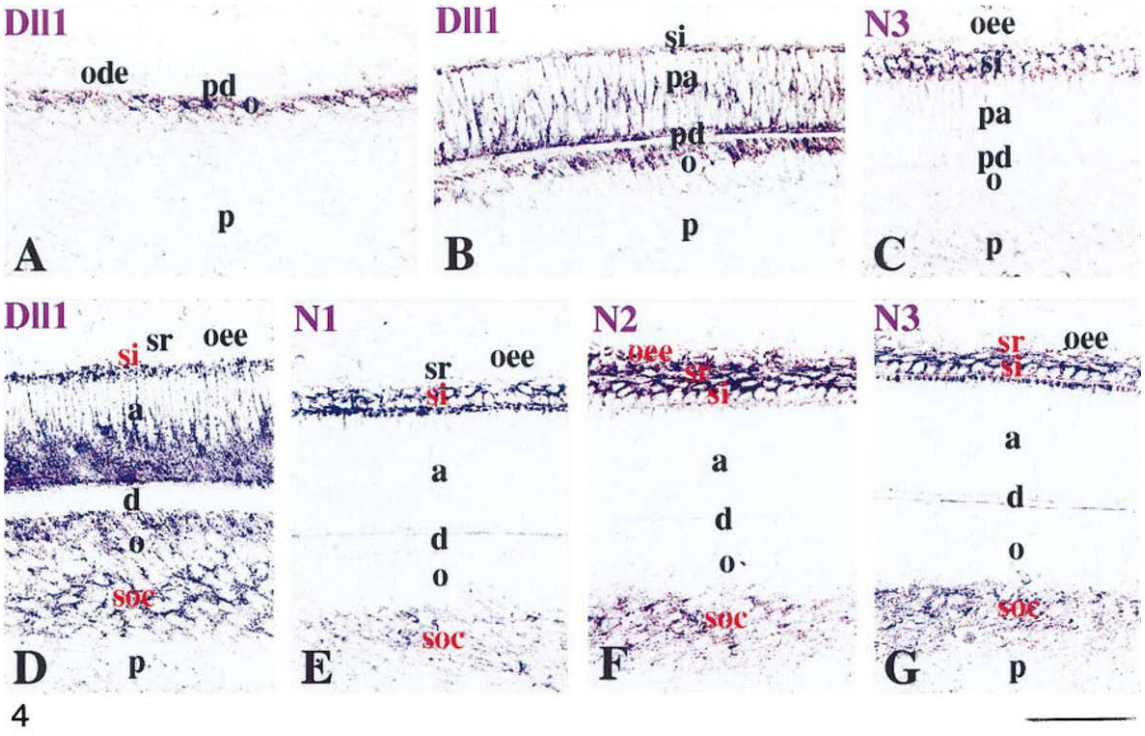
Induction of Notch1 and Notch2 Expression by Dll1-Expressing Cells

In the developing incisor, *Dll1* expression in differentiating odontoblasts appears to precede the upregulation of *Notch1, Notch2,* and *Notch3* in the adjacent cells of the dental papilla that will form the subodontoblastic layer (Figs. 3B, 4B, and 4C). This observation suggests that *Dll1* expression may play a role in upregulating expression of Notch receptors in neighboring cells, a possibility supported by studies in *Drosophila,* in which forced expression of Delta in the wing leads to increased *Notch* expression in adjacent cells (Huppert *et al.*, 1997). If this hypothesis is correct, *Dll1*-expressing cells should upregulate expression of Notch receptors in cocultured dental mesenchyme *in vitro.* We thus cocultured *cDll1*-expressing QT6 cells with E16.5 dental explants. After coculture for 24 h, the *cDll1*-expressing QT6 cells were identified by *in situ* hybridization using a *cDll1* digoxigenin-labeled probe, while the Notch1- and Notch2-positive cells (red color) were visualized by immunohistochemistry using polyclonal anti-Notch antisera (Figs. 5A and 5B). The cells surrounding the implanted *cDll1*-expressing cells were strongly stained by both anti-Notch1 (Fig. 5A) and anti-Notch2 (Fig. 5B) antisera. In contrast, immunoreactivity with anti-Notch3 antisera was not induced (data not shown). Only low levels of Notch1 and Notch2 protein expression were seen in the mesenchymal cells farther away from the implanted cells. Untransfected QT6 cells had no effect on Notch1 and Notch2 expression in dental mesenchyme (Figs. 5C and 5D). These results suggest that feedback regulations between Notch ligands and receptors may be involved in setting up the complementary *Dll1*- and *Notch*-expressing cell layers in the developing tooth.

Dll1 Expression Is Not Affected by Epithelial–Mesenchymal Interactions

Since Notch receptors and the Jag1 ligand are regulated by epithelial–mesenchymal interactions during tooth development (Mitsiadis *et al.*, 1995, 1997), we asked whether this might also be true for *Dll1*. Dissected pieces of E12.5–E16.5 mouse dental epithelia and mesenchyme of the molar region were cultured as isochronic or het-

FIG. 4. Comparison between *Dll1, N1, N2,* and *N3* mRNA expression in selected areas of an E18.5 lower incisor. (A) Anterior part of the lingual side, (B and C) posterior part of the labial side, (D–G) anterior part of the labial side. (A) *Dll1* mRNA expression in differentiating odontoblasts (o) of the lingual side. Note the absence of expression in the dental papilla (p). (B) At the labial side of the incisor (posterior part), preameloblasts (pa) and odontoblasts (o) express *Dll1,* while *N3* expression is restricted to the stratum intermedium (si) (C). Some *N3* transcripts are also seen in cells of the dental papilla (p). (D) Intense *Dll1* expression is observed in ameloblasts (a), odontoblasts, and cells of the subodontoblastic layer (soc) of the labial side (anterior part). (E–G) In adjacent sections, note the coexpression of *N1, N2,* and *N3* in cells of the stratum intermedium and the subodontoblastic layer. *N2* is also expressed in the stellate reticulum (sr) and the outer enamel epithelium (oee). Expression is not observed in ameloblasts and odontoblasts. Other abbreviations as in Fig. 3. Size bar, 50 μm.



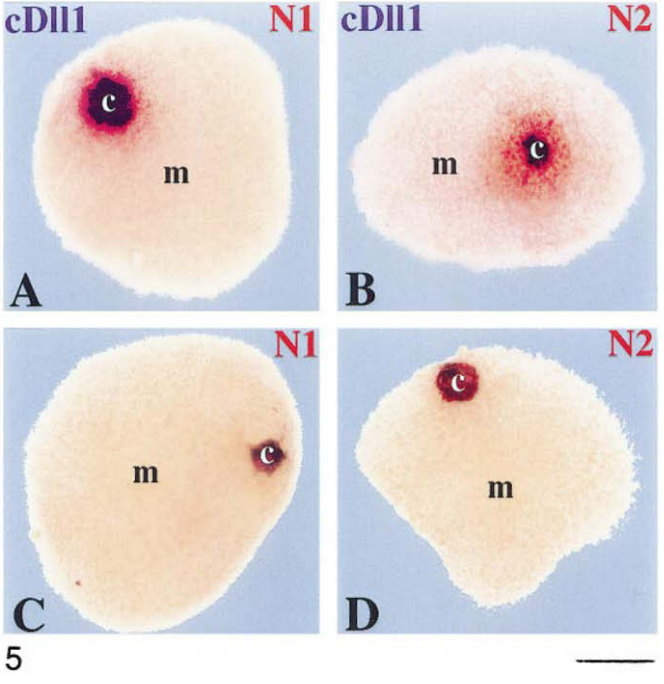


FIG. 5. Coculturing QT6 cells expressing chick *Dll1* (*cDll1*) with E16.5 dental mesenchyme leads to Notch upregulation in the mesenchyme. *In situ* hybridization using digoxigenin-labeled *cDll1* (A, B) or chick *MyoD* (C, D) was combined with immunohistochemistry using polyclonal anti-N1 or anti-N2 antisera. Explants were cocultured for 24 h with QT6 cells. (A and B) QT6 cells expressing *cDll1* (violet) strongly upregulate N1 and N2 expression (red) in adjacent mesenchymal cells. (C and D) Untransfected QT6 cells visualized with a chick *MyoD* probe showed no effect on N1 and N2 expression in dental mesenchyme. Note the faint N1 and N2 immunostaining throughout the mesenchyme. Size bar, 100 μm.

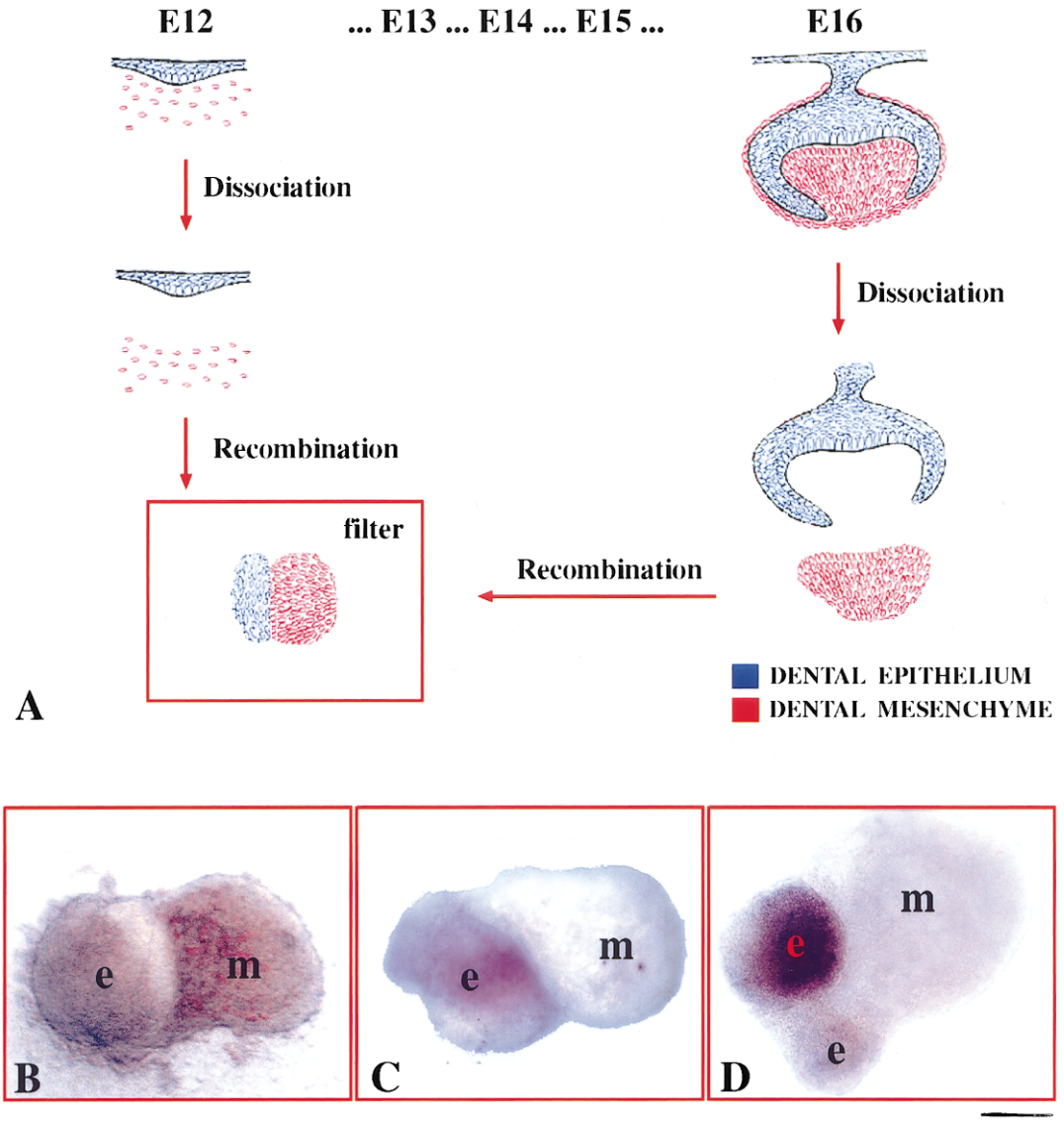


FIG. 6. Expression of *Dll1* mRNA in explants of recombinants between dental epithelium and mesenchyme of different stages. Whole-mount *in situ* hybridizations using the digoxigenin-labeled *Dll1* probe are shown. (A) The design of experiments used to analyze tissue interactions *in vitro*. The dental epithelium (blue) and mesenchyme (red) are separated and cultured in recombination on a filter. (B) Photomicrograph of an unstained explant of an E14.5 tooth epithelium (e) recombined with an E14.5 dental mesenchyme (m) after 24 h of culture. Note the presence of a translucent zone in the epithelium (epithelial–mesenchymal interface), indicating the inductive capacity of the dental mesenchyme during this stage (Vainio *et al.*, 1993). (C) *Dll1* is expressed only in the epithelium of the same isochronic recombinant. (D) Expression of *Dll1* in a recombinant of an E16.5 mesenchyme with an E16.5 (upper, red letter) and an E12.5 (lower, black letter) epithelium. While *Dll1* is expressed in both epithelia, intense expression is observed only in E16.5 epithelial cells. Note the faint *Dll1* expression in the mesenchyme at this stage. Size bar, 100 μm.

erochronic recombinants (Fig. 6A) and then analyzed for *Dll1* expression by whole-mount *in situ* hybridization. As expected, *Dll1* expression is found in the epithelial part of the recombinants: very weak in cultured E12.5 epithelia (Fig. 6D), *Dll1* expression became clearly visible in E14.5 epithelium (Fig. 6C) and even stronger in the E16.5 epithelium (Fig. 6D). In heterochronic recombinants, *Dll1* expression in the E12.5 epithelium was not affected by E16.5 mesenchyme (Fig. 6D), indicating that the upregulation of *Dll1* expression in dental epithelium, which occurs at the early bell stage, is independent of mesenchyme-derived signals. Similarly, *Dll1* expression in the mesenchyme was not induced by epithelium in any recombination tested (Figs. 6C and 6D).

TGFβ1 and BMPs Induce Dll1 Expression in Dental Mesenchyme during Specific Developmental Periods

It has been shown previously that members of the TGFβ superfamily are involved in odontoblast differentiation (for a review see Ruch *et al.*, 1995, and references therein), suggesting that these signaling molecules may play a role in the regulation of *Dll1* expression in the mesenchyme. To test this, we placed beads releasing TGFβ1 and BMP4 on top of E16.5 dental explants and followed the expression of *Dll1* by whole-mount *in situ* hybridization. Analysis of the explants shows that *Dll1* is upregulated in mesenchymal cells surrounding beads containing either BMP4 (Figs. 7A and 7D) or TGFβ1 (Figs. 7B and 7E). BMP4 induced *Dll1* expression in a wide area of cells surrounding the bead, while the effect was much more restricted after local application of TGFβ1. By contrast, when the BMP4- or TGFβ1-releasing beads were placed on top of E13.5 explants, *Dll1* expression was not induced in the surrounding cells (data not shown), showing that early mesenchyme is unable to respond. We showed recently that FGF4 upregulates *Jag1* expression in dental mesenchyme explants (Mitsiadis *et al.*, 1997). In contrast, *Dll1* expression was not affected by beads releasing FGF4 in E16.5 dental explants (Fig. 7C). As a control, we used beads soaked in BSA and in no case was *Dll1* expression induced (Fig. 7F).

DISCUSSION

Delta1 and Notch Receptor Genes Are Expressed in Adjacent Cell Populations

Notch signaling controls cell commitment in a wide range of tissues and organs, in both invertebrates and vertebrates (Artavanis-Tsakonas *et al.*, 1995). The interaction between Dll1 and Notch family members requires their concomitant expression in adjacent cells. During the cytodifferentiation stage of mouse odontogenesis, *Dll1* and the three *Notch* genes analyzed show complementary expression patterns at several sites. *Dll1* is expressed in differentiating odontoblasts and ameloblasts, whereas expression of the *Notch* genes is confined mainly to the subodontoblastic layer and the stratum intermedium which underlies the ameloblast layer. This expression of *Notch* receptors and *Dll1* by two juxtaposed cell populations suggests a role for Delta–Notch signaling in controlling odontoblast and ameloblast differentiation. For example, in dental mesenchyme, preodontoblasts give rise to odontoblasts and cells of the subodontoblastic layer. One interpretation is thus that expression of *Dll1* in newborn odontoblasts directs the adjacent cells toward an alternative fate (i.e., cells of the subodontoblastic layer). However, control of such cell fate choices by Notch signaling has not yet been reported in vertebrates. Rather, results obtained on neuronal differentiation in the *Xenopus* neural plate (Chitnis *et al.*, 1995) and in the chick (Henrique *et al.*, 1997) and *Xenopus* (Dorsky *et al.*, 1997) retina show that cells exposed to Dll1 signaling are prevented from exiting the mitotic cycle and from differentiating, instead of being driven to adopt a specific differentiated state. In line with this, an alternative interpretation of our results is that expression of *Dll1* in newborn odontoblasts inhibits the adjacent cells from exiting the cell cycle, thus providing a feedback mechanism to control the proportion of cells that will differentiate into odontoblasts. On the epithelial side, Delta–Notch signaling between preameloblasts/ameloblasts and the adjacent stratum intermedium may prevent stratum intermedium cells from adopting an ameloblast fate. As in neurogenesis, Delta–Notch signaling may ensure a continuous supply of progenitors, necessary for molar and incisor growth during development and for continuous replacement of incisors in the mouse by preventing premature differentiation.

In the incisor, the most posterior part of the epithelium (or cervical loop) represents a reservoir of immature cells which will generate the four cell layers according to a posterioanterior gradient of cytodifferentiation. *Notch3* is expressed in all epithelial cells of the cervical loop, but more anteriorly, its expression is downregulated in cells destined to become ameloblasts and restricted to cells of the stratum intermedium, while *Notch1* is expressed only in the cells that will form the stratum intermedium. In contrast, *Dll1* expression in the cervical loop is confined to a subpopulation of cells in continuity with the layer of cells that gives rise to ameloblasts. The complementary expression patterns of *Notch* receptors and *Dll1* during incisor differentiation suggests that, as in other systems (Lewis, 1996), Notch receptors and Delta ligands may act to generate differences between initially equivalent cells in the incisor epithelium.

In vertebrates, there are four different *Notch* genes and at least as many ligands, and the question of which ligand interacts with which receptor has not been resolved (Weinmaster, 1997). Our results showing *Dll1* expression in cells adjacent to cells that express *Notch1*, *2*, and/or *3* support the possibility that this ligand can interact with any of the three receptors. On the other hand, activation of different Notch receptors by Dll1 may not be identical. In line with this, Jag1 is more effective than Dll1 in activating Notch2, while both Jag1 and Dll1 can activate Notch1 efficiently (Lindsell *et al.*, 1995; Weinmaster, 1997). The *Dll1* expression pattern presented here contrasts with that of the other Notch ligand Jag1, which is expressed during early tooth morphogenesis in both the epithelium and the mesenchyme, preceding that of *Dll1* (Mitsiadis *et al.*, 1997). *Jag1* transcripts disappear from the dental mesenchyme at the early bell stage and persist in the epithelial components only in the stratum intermedium at the stage at which *Dll1* is upregulated in the dental epithelium. The differential expression patterns of *Dll1* and *Jag1* in the developing tooth argue for distinct roles for these two ligands and suggest that specific ligand–receptor pairs which regulate different fate choices may exist during odontogenesis.

Delta Exerts Feedback Regulation on Notch1 and Notch2 Expression in Dental Mesenchyme

A striking feature of the *Notch* and *Dll1* expression patterns in the tooth germ is that they are mainly complementary and confined to different cell layers. This contrasts with most of the well-studied models of Delta–Notch signaling, such as the *Drosophila* neuroectoderm (Artavanis-Tsakonas *et al.,* 1991) and the vertebrate neural tube and retina (Austin *et al.,* 1995; Chitnis *et al.,* 1995; Henrique *et al.,* 1995, 1997; de la Pompa *et al.,* 1997; Dorsky *et al.,* 1997), where the *Notch*- and *Delta*-expressing cells are intermingled. However, a similar situation is encountered during pupal wing vein formation in *Drosophila,* where *Delta* and *Notch* are also expressed in complementary patterns in adjacent stripes of cells (Huppert *et al.,* 1997). In this system, accumulation of Notch in intervein cells depends on Delta signaling, and constitutive Notch signaling represses *Delta* expression in presumptive vein cells. Such feedback regulations exerted by Delta–Notch signaling may also be responsible for the asymmetries in ligand and receptor expression in the tooth germ, since we could show that *Dll1*-expressing cells upregulate Notch1 and Notch2 expression in adjacent cells in explants of dental mesenchyme. Feedback regulation of *Delta* and *Notch* expression by Delta–Notch signaling has also been inferred from somatic mosaic analysis in *Drosophila* (Heitzel and Simpson, 1991), but evidence for the existence of such regulatory loops in vertebrates has been lacking. Positive regulation of Notch1 and Notch2 expression and negative regulation of Dll1 expression by Delta–Notch signaling may thus ensure that *Notch1, 2,* and *3* and *Dll1* expression are kept segregated in different cell layers.

Regulation of Dll1 Expression in Dental Mesenchyme

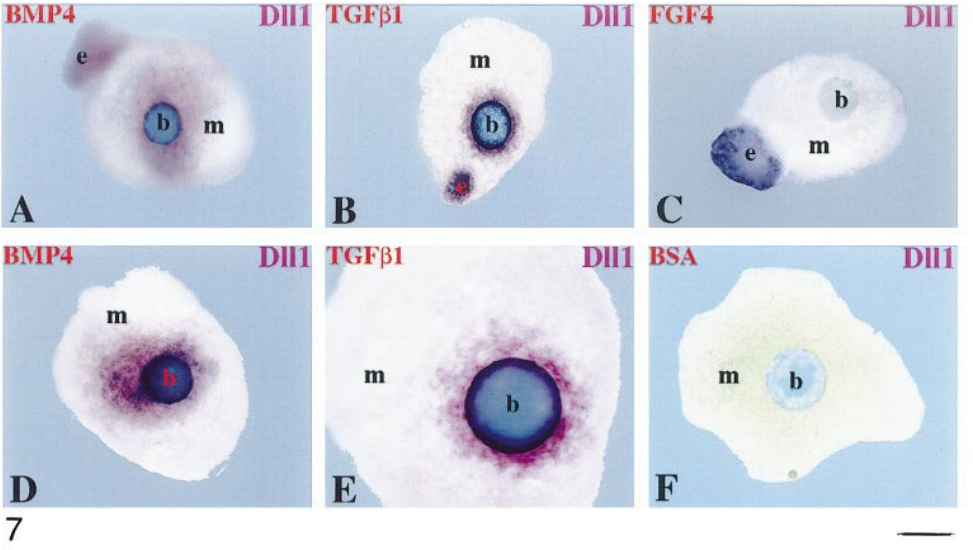
A complex series of sequential and reciprocal interactions between the oral epithelium and the neural crest-derived mesenchyme govern tooth initiation and morphogenesis (for review, see Thesleff, 1995). In contrast to the expression of *Jag1* (Mitsiadis *et al.,* 1997) and of the Notch receptors (Mitsiadis *et al.,* 1995), expression of *Dll1* was not affected by epithelio-mesenchymal interactions in dental explants, suggesting that signals intrinsic to both epithelium and mesenchyme are responsible for inducing *Dll1* in dental tissues. This is in agreement with recent results showing the autonomous property of the chick presomitic mesoderm to maintain the *in vivo* expression pattern of chick *Dll1* (Palmeirim *et al.,* 1988).

Signaling molecules of the TGFβ superfamily are good candidates for being involved in autocrine signaling within the dental mesenchyme. During the bell stage of molar development, BMP2, BMP4 (Vainio *et al.,* 1993; Åberg *et al.,* 1997), and TGFβ1 (Vaahtokari *et al.,* 1991) are expressed in preodontoblasts/odontoblasts. Furthermore, TGFβ1 and BMP2 have been shown to induce odontoblast differentiation *in vitro* (reviewed by Ruch *et al.,* 1995). We here show that both BMP4 and TGFβ1 induce *Dll1* expression in the mesenchyme when applied to E16.5 dental explants. However, the *Bmp4* gene is already expressed in the dental papilla at the early bell stage, well before *Dll1* expression in the mesenchyme and odontoblast differentiation have started. The use of the same signals for many different decisions implies that periods of active signaling must coincide with distinct periods of competence in the responding cells. Early progenitors cannot generate odontoblasts, suggesting that the acquisition of the odontoblast fate depends on changes in both progenitor competence and the action of signaling molecules. Indeed, *Dll1* expression was induced by BMP4 and TGFβ1 in E16.5, but not in E13.5 dental mesenchyme, showing that changes in the cells' responsiveness to signaling molecules have occurred over this time period. Intrinsic properties of progenitors have been proposed to control the time of generation of different cell types also in other systems (Watanabe and Raff, 1990).

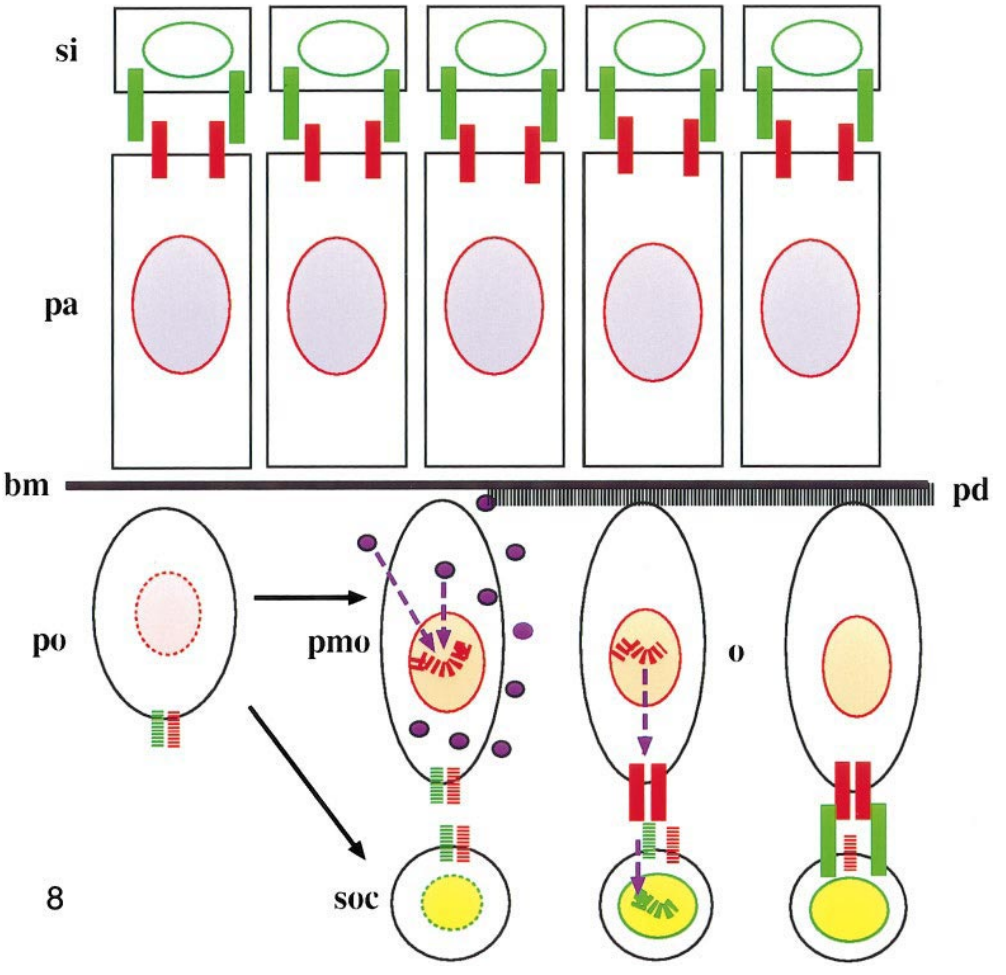
In conclusion, the data presented here show that during tooth development both the expression patterns and the regulatory mechanisms for the Notch receptors and their two ligands Jag1 and Dll1 differ and suggest a feedback

FIG. 7. Effects of signaling molecules on *Dll1* expression in dental mesenchyme explants. The explants were cultured for 24 h. Whole-mount *in situ* hybridizations using the digoxigenin-labeled Dll1 probe are shown. (A–C) E16.5 dental epithelia were cultured together with isochronic dental mesenchyme, in which beads soaked in 100 μg/ml BMP4, TGFβ1, or FGF4 were implanted. *Dll1* transcripts (violet) are found in the epithelium and in mesenchymal cells surrounding the TGFβ1 and BMP4 beads, while the FGF4 bead does not induce *Dll1* expression. (D–F) Isolated E16.5 dental mesenchyme was cultured with implanted TGFβ1, BMP4, and BSA beads. *Dll1* expression is observed in cells surrounding the TGFβ1 and BMP4 beads, while the BSA bead does not induce *Dll1* expression. Size bar, (A–D, F) 100 μm; (E) 40 μm.

FIG. 8. Schematic representation of the expression patterns of Notch family receptors (bars in green) and their Dll1 ligand (bars in red) in the tooth during cytodifferentiation events. A working model to describe the activation of Delta–Notch signaling during odontoblast differentiation is presented. Preodontoblasts (po) express minimal levels (disconnected green and red bars) of both Notch receptors and the Dll1 ligand before they give rise to postmitotic polarizing odontoblasts (pmo) and cells of the subodontoblastic layer (soc). The postmitotic polarizing odontoblasts produce molecules of the TGFβ superfamily (i.e., TGFβ1 and BMPs; small circles in violet) which increase their levels of Dll1 expression. The postmitotic polarizing odontoblasts become functional odontoblasts (o) and begin to secrete predentine (pd). The Dll1 ligand expressed by functional odontoblasts upregulates the expression of Notch receptors in cells of the subodontoblastic layer, thereby maintaining the segregation of Notch receptors and the Dll1 ligand in different cell layers. Other abbreviations: bm, basement membrane; pa, preameloblasts; si, stratum intermedium.



7



8

regulation by Delta–Notch signaling to ensure the spatial segregation of Notch receptors and ligands (Fig. 8).

ACKNOWLEDGMENTS

The authors thank Dr. I. Thesleff (University of Heslinki, Finland) for the use of her laboratory facilities during a short-term visit (T.M.). We are grateful to Dr. O. Pourquié for the chick QT6 cell transformants and to Dr. D. Henrique for the *Dll1* and *cDll1* probes. We are indebted to Drs. C. Henderson (IBDM, Marseille, France) and S. Artavanis-Tsakonas (Yale University, New Haven, CT) for critically reading the manuscript, and to Dr. J. Lewis (Imperial Cancer Research Fund, London, UK) for stimulating discussions and advice. This work was supported by grants from the Fondation pour la Recherche Médicale (T.M.), the Association pour la Recherche sur le Cancer, the Ligue Nationale Contre le Cancer (T.M., C.G.), and the Swedish Medical Research Council (U.L.) and by short-term fellowships from EMBO and the European Science Foundation (T.M.).

REFERENCES

Åberg, T., Wozney, J., and Thesleff, I. (1997). Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn.* **210,** 383–396.

Artavanis-Tsakonas, S., Delidakis, C., and Fehon, R. G. (1991). The *Notch* locus and the cell biology of neuroblast segregation. *Annu. Rev. Cell Biol.* **7,** 427–452.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch signaling. *Science* **268,** 225–232.

Bettenhausen, B., Hrabé de Angelis, M., Simon, D., Guénet, J.-L., and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely related to *Drosophila Delta*. *Development* **121,** 2407–2418.

Conlon, R. A., Reaume, A. G., and Rossant, J. (1995). *Notch 1* is required for the coordinate segmentation of somites. *Development* **121,** 1533–1545.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375,** 761–766.

Crowe, R., and Niswander, L. (1998). Disruption of scale development by *Delta-1* misexpression. *Dev. Biol.* **195,** 70–74.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J., and Conlon, R. A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124,** 1139–1148.

Dorsky, R. I., Chang, W. S., Rapaport, D. H., and Harris, W. A. (1997). Regulation of neuronal diversity in the *Xenopus* retina by Delta signalling. *Nature* **385,** 67–70.

Gu, Y., Hukriede, N. A., and Fleming, R. J. (1995). Serrate expression can functionally replace Delta activity during neuroblast segregation in the *Drosophila* embryo. *Development* **121,** 855–865.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in *Drosophila* neuroepithelium. *Cell* **64,** 1083–1092.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a *Delta* homologue in prospective neurons in the chick. *Nature* **375,** 787–790.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquié, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta–Notch signalling in the embryonic chick retina. *Curr. Biol.* **7,** 661–670.

Huppert, S. S., Jacobsen, T. L., and Muskavitch, M. A. (1997). Feedback regulation is central to Delta–Notch signalling required for *Drosophila* wing vein morphogenesis. *Development* **124,** 3283–3291.

Kopan, R., Nye, J. S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: A constitutively activated repressor of myogenesis directed at the basic helix–loop–helix region of MyoD. *Development* **120,** 2385–2396.

Kopan, R., and Weintraub, H. (1993). Mouse *Notch:* Expression in hair follicles correlates with cell fate determination. *J. Cell Biol.* **121,** 631–641.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6,** 3–10.

Lindsell, C. E., Shawber, C. J., Boulter, J., and Weinmaster, G. (1995). Jagged: A mammalian ligand that activates Notch 1. *Cell* **80,** 909–917.

Mitsiadis, T. A., Henrique, D., Thesleff, I., and Lendahl, U. (1997). Mouse *Serrate-1* (*Jagged-1*): Expression in the developing tooth is regulated by epithelial–mesenchymal interactions and fibroblast growth factor-4. *Development* **124,** 1473–1483.

Mitsiadis, T. A., Lardelli, M., Lendahl, U., and Thesleff, I. (1995). Expression of *Notch 1, 2* and *3* is regulated by epithelial–mesenchymal interactions and retinoic acid in the developing mouse tooth and associated with determination of ameloblast cell fate. *J. Cell Biol.* **130,** 407–418.

Nye, J. S., and Kopan, R. (1995). Vertebrate ligands for Notch. *Curr. Biol.* **5,** 966–969.

Palmeirim, I., Dubrulle, J., Henrique, D., Ish-Horowicz, D., and Pourquié, O. (1998). Uncoupling segmentation and somitogenesis in the chick presomitic mesoderm. *Dev. Genet.* **23,** 77–85.

Pourquié, O., Fan, C.-M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: A role for BMP4. *Cell* **84,** 461–471.

Ruch, J.-V., Lesot, H., and Bègue-Kirn, C. (1995). Odontoblast differentiation. *Int. J. Dev. Biol.* **39,** 51–68.

Simpson, P. (1995). The Notch connection. *Nature* **375,** 736–737.

Sun, X., and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the *Drosophila* Notch ligands. *Development* **122,** 2465–2474.

Thesleff, I., Vaahtokari, A., and Partanen, A.-M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39,** 35–50.

Vaahtokari, A., Vainio, S., and Thesleff, I. (1991). Associations between transforming growth factor β1 RNA expression and epithelial–mesenchymal interactions during tooth morphogenesis. *Development* **113,** 985–994.

Vainio, S., Karavanova, I., Jowett, A., and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75,** 45–58.

Watanabe, T., and Raff, M. C. (1990). Rod photoreceptor development in vitro: Intrinsic properties of proliferating neuroepithelial cells change as development proceeds in the rat retina. *Neuron* **4,** 461–467.

Weinmaster, G. (1997). The ins and outs of Notch signaling. *Mol. Cell. Neurosci.* **9,** 91–102.

Received for publication July 17, 1998
Revised September 17, 1998
Accepted September 17, 1998