

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

Thimos A. Mitsiadis,^{**} Michael Lardelli,[‡] Urban Lendahl,[‡] and Irma Thesleff^{*}

^{*}Department of Pedodontics and Orthodontics, Institute of Dentistry, University of Helsinki, FIN-000 14 Helsinki, Finland; and

[‡]Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, S-171 77 Stockholm, Sweden

Abstract. *Notch 1*, *Notch 2*, and *Notch 3* are three highly conserved mammalian homologues of the *Drosophila Notch* gene, which encodes a transmembrane protein important for various cell fate decisions during development. Little is yet known about regulation of mammalian *Notch* gene expression, and this issue has been addressed in the developing rodent tooth during normal morphogenesis and after experimental manipulation. *Notch 1*, *2*, and *3* genes show distinct cell-type specific expression patterns. Most notably, *Notch* expression is absent in epithelial cells in close contact with mesenchyme, which may be important for acquisition of the ameloblast fate. This reveals a previously unknown prepatterning of dental epithelium at early stages, and suggests that mesenchyme negatively regulates *Notch* expression in epithelium. This hypothesis

has been tested in homo- and heterotypic explant experiments in vitro. The data show that *Notch* expression is downregulated in dental epithelial cells juxtaposed to mesenchyme, indicating that dental epithelium needs a mesenchyme-derived signal in order to maintain the downregulation of *Notch*. Finally, *Notch* expression in dental mesenchyme is upregulated in a region surrounding beads soaked in retinoic acid (50–100 $\mu\text{g/ml}$) but not in fibroblast growth factor-2 (100–250 $\mu\text{g/ml}$). The response to retinoic acid was seen in explants of 11–12-d old mouse embryos but not in older embryos. These data suggest that *Notch* genes may be involved in mediating some of the biological effects of retinoic acid during normal development and after teratogenic exposure.

THE development of most organs in vertebrates depends on a complex set of inductive interactions between epithelium and mesenchyme. These sequential and reciprocal interactions lead to the determination of cell fate and the organization of cells into tissues and organs. In the developing tooth, changes in gene expression patterns of several growth factors, transcription factors, cell surface molecules, and structural molecules of the extracellular matrix have been implicated during the progressive determination of epithelial and mesenchymal cells (Vainio et al., 1989, 1993; Mitsiadis et al., 1992, 1995a,b; Mitsiadis and Luukko, 1995; Thesleff et al., 1992, 1995).

The *Drosophila Notch* gene encodes a large transmembrane receptor controlling cell fate decisions from clusters of cells with equivalent developmental potential in many tissues, such as the nervous system and muscle (Artavanis-

Tsakonas et al., 1991; Corbin et al., 1991; Fortini and Artavanis-Tsakonas, 1993). On the extracellular side Notch contains 36 epidermal growth factor (EGF)-like repeats and three *Notch/lin-12* repeats, while the intracellular domain contains six copies of the ankyrin repeat, a motif important for cell signaling (Artavanis-Tsakonas and Simpson, 1991; Rebay et al., 1993; Struhl et al., 1993; Greenwald, 1994). Loss of the *Notch* gene results in overcommitment of ectodermal cells to the neural fate at the expense of epidermal cell formation in the fly larva. Analysis of a variety of mutant *Notch* alleles has shown that Notch is also involved in differentiation of many other tissues such as the somatic follicle cells, the eye and wing (de Cellis et al., 1991; Fortini et al., 1993; Rebay et al., 1993; Cummings and Cronmiller, 1994). Delta and Serrate are ligands for Notch, and bind directly to specific EGF repeats of the Notch receptor (Fehon et al., 1990; Rebay et al., 1991).

Recently three highly conserved vertebrate homologues of the *Drosophila Notch* gene have been characterized: *Notch 1*, *2*, and *3* (for reviews and references see Greenwald, 1994; Lardelli et al., 1995). Mutations in *Notch* genes result in dramatic developmental effects in vertebrates. Truncation of the human *Notch 1* gene by chromosomal

Address all correspondence to T. A. Mitsiadis, Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, S-171 77 Stockholm, Sweden. Tel.: 46 8 7287353. Fax: 46 8 348135.

The current address of M. Lardelli is Department of Developmental Neuroscience, Biomedical Center, University of Uppsala, Box 587, S751 23 Uppsala, Sweden.

translocation is associated with leukemia (Ellisen et al., 1991) and similar truncations of the *Notch*-related gene *int-3* produces breast tumors in mice (Jhappan et al., 1992; Robbins et al., 1992). Inactivation of the mouse *Notch 1* gene by gene targeting results in a lethal phenotype at embryonic day-11.5 (E11.5) (Swiatek et al., 1994) and inactivation of the *Notch 2* gene leads to perinatal death, probably due to kidney failure (Gridley, T., personal communication). Furthermore, injection of RNA encoding a truncated *Notch 1* gene in *Xenopus* produces excessive neural tube development (Coffman et al., 1993).

Relatively little is yet known about regulation of *Notch* gene expression. The developing rodent tooth is a useful model for examination of the molecular mechanisms involved in gradual cell fate determination and the differentiation of various cell lineages (Thesleff et al., 1992, 1995), and has many advantages to study experimentally the influence of tissue interactions and growth factors on gene regulation (Vainio et al., 1993; Mitsiadis et al., 1995a). Tooth development results from sequential and reciprocal interactions between cranial neural crest-derived mesenchyme and the oral ectoderm (Thesleff and Hurmerinta, 1981; Lumsden, 1988). The first morphological signs of tooth development are local thickenings in the stomodeal epithelium of the E11 mouse embryo. The presumptive dental epithelium then invades the underlying jaw mesenchyme forming a bud by E13, around which the mesenchymal cells condense. Cells of the dental papilla mesenchyme lying directly under the epithelium differentiate into odontoblasts that secrete the organic matrix of dentin. Epithelial cells in close contact with the dental mesenchyme differentiate into ameloblasts which produce the enamel matrix. Inductive interactions can be studied in dissected pieces of dental epithelium and mesenchyme cultured as recombinants. This system also allows for studying gene regulation with beads releasing growth factors and signaling molecules. The effects of retinoic acid (RA)¹, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and midkine (MK) on cell proliferation and the expression of a number of genes have been analyzed in this way (Vainio et al., 1993; Jernvall et al., 1994; Mitsiadis et al., 1995a).

In this report we examine the expression of the *Notch 1*, *2*, and *3* genes in the developing tooth during normal development and in recombination explants of epithelium and mesenchyme. We also test the effects of cell-signaling molecules on the expression of *Notch* in dental tissue explants. The data presented show that the three *Notch* genes exhibit distinct expression patterns in developing teeth and that they are regulated by epithelial-mesenchymal interactions and RA.

Materials and Methods

Animals and Tissue Preparation

F1(CBA x C57BL or CBA x NMRI) mice were used at embryonic and postnatal stages. The age of the mouse embryos was determined according

1. Abbreviations used in this paper: BMP, bone morphogenetic protein; MK, midkine; RA, retinoic acid.

to the vaginal plug (day 0) and confirmed by morphological criteria. Animals were sacrificed by cervical dislocation and the embryos were surgically removed. The heads from the embryonic day-11 (E11) mouse embryos to the postnatal day-8 (PN8) pups were dissected in Dulbecco's phosphate-buffered saline, pH 7.4. Jaws and mandibular molar tooth germs were removed, fixed overnight at 4°C with paraformaldehyde (PFA) in PBS. After dehydration the tissues were embedded in paraffin wax, and serially sectioned at 5 µm on silanized slides, dried overnight, and stored in tight boxes at 4°C.

Probes and Antibodies

Probes for the three *Notch* genes were, in each case, derived from a region encoding the last six EGF-repeats and the Notch/lin-12 repeats of the three *Notch* genes, as previously described (Lardelli et al., 1994; Larsson et al., 1994). After linearization of the plasmid vectors, single-stranded sense Notch 1 (pT3), Notch 2 and 3 (pT7), and antisense Notch 1 (pT7), Notch 2 and 3 (pT3) cRNA probes were prepared by standard procedures, as previously described (Mitsiadis et al., 1995b).

Polyclonal antisera to the Notch 1 and 2 proteins were generated in the following way. DNA fragments corresponding to amino acids 1274-1465 from the *Notch 1* and 1280-1474 from the *Notch 2* gene were PCR amplified, sequenced, and cloned into the bacterial expression vector pEZZ18 to produce a fusion protein with the Z region of *Staphylococcus aureus* protein A. The fusion protein was harvested and purified from *E. coli* on an IgG column and used to immunize rabbits (Löwenadler et al., 1987). Booster immunizations were given at 3 and 6 wk, and the antisera harvested at 24 wk, after the initial immunization.

In Situ Hybridization and Immunohistochemistry on Tissue Sections

In situ hybridization on paraffin sections using [³⁵S]O₄-UTP-labeled (Amersham Corp., Arlington Heights, IL) single-stranded RNA probes was performed as described previously (Mitsiadis et al., 1995a,b). For immunohistochemistry, the deparaffinized sections were preincubated with 0.3% H₂O₂ in absolute methanol (Mitsiadis et al., 1995a,b), followed by an overnight incubation at 4°C with Notch 1 or Notch 2 antiserum (dilutions 1:800 and 1:1,000, respectively) in 2% normal goat serum (NGS)/0.2% BSA in PBS, pH 7.4. Control sections were incubated either with normal rabbit serum (NRS) or with 2% BSA in PBS. After several washes the sections were incubated with biotinylated secondary goat anti-rabbit antibody (dilution 1:250 in PBS), washed again and incubated with avidin-biotin-peroxidase complex (Vector VECTASTAIN Elite ABC kit; Vector Laboratories Inc., Burlingame, CA). Peroxidase enzyme activity was visualized by incubation with 3-amino-9-ethylcarbazole (AEC). Controls for Notch 1 and 2 antiserum specificity were performed by adsorbing the antisera for 16 h at 4°C with 1.14 mg/ml and 0.42 mg/ml of the fusion proteins, which had been preincubated in a solution of 3% human IgG and 0.25% gelatin in PBS for 1 h at 37°C. The slides were then washed and developed as described above.

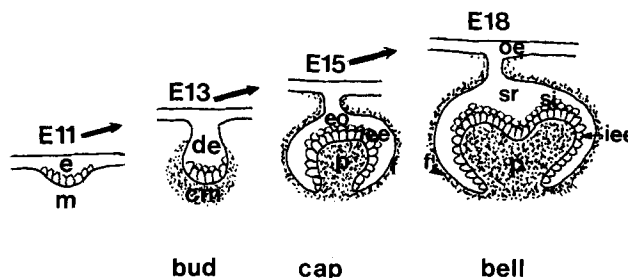


Figure 1. Schematic representation of the successive stages of odontogenesis of the mouse molar. Abbreviations: e, presumptive dental epithelium; m, mesenchyme; de, dental epithelium; cm, condensed mesenchyme; eo, enamel organ; iee, inner enamel epithelium; p, dental papilla mesenchyme; sr, stellate reticulum; si, stratum intermedium; oe, oral epithelium; f, dental follicle.

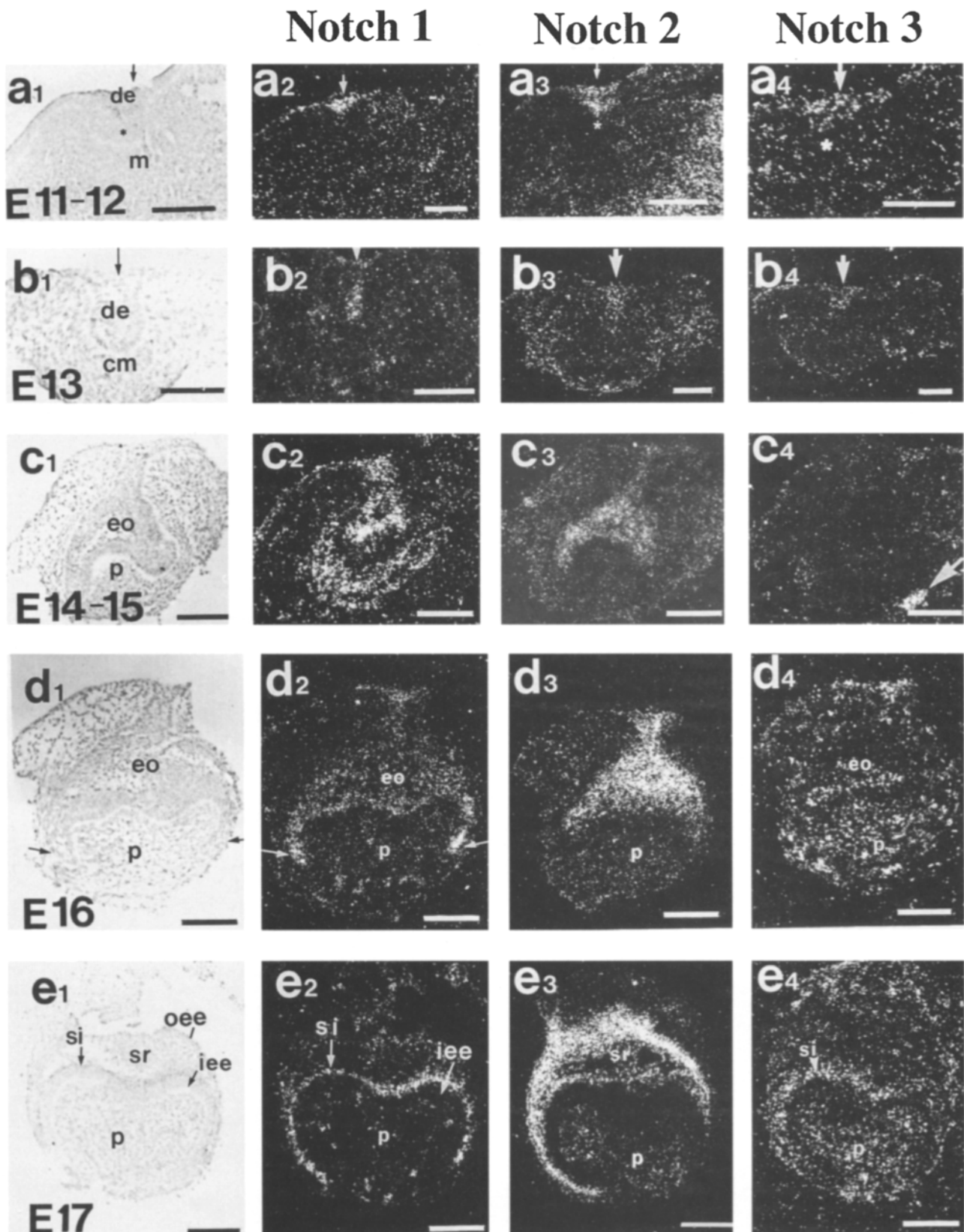


Figure 2. Localization of *Notch 1*, *2*, and *3* mRNAs by in situ hybridization in embryonic mouse molar tooth germs (E11-E17). (*x1*) bright-field micrographs; (*x2*, *x3*, and *x4*) dark-field micrographs. (*a*) At E11-E12, *Notch* transcripts are intensely expressed in the thickened dental epithelium (*de*, arrow), but are absent from cells of the basal layer (asterisk). (*b*) All three *Notch* genes are expressed in epithelium of the bud staged tooth (E13). Transcripts are absent from cells of its basal layer (asterisk) and condensed mesenchyme (*cm*). (*c*) During the cap stage (E14-E15), *Notch 1* and *2* mRNAs are expressed in cells of the enamel organ (*eo*), except in cells of the basal

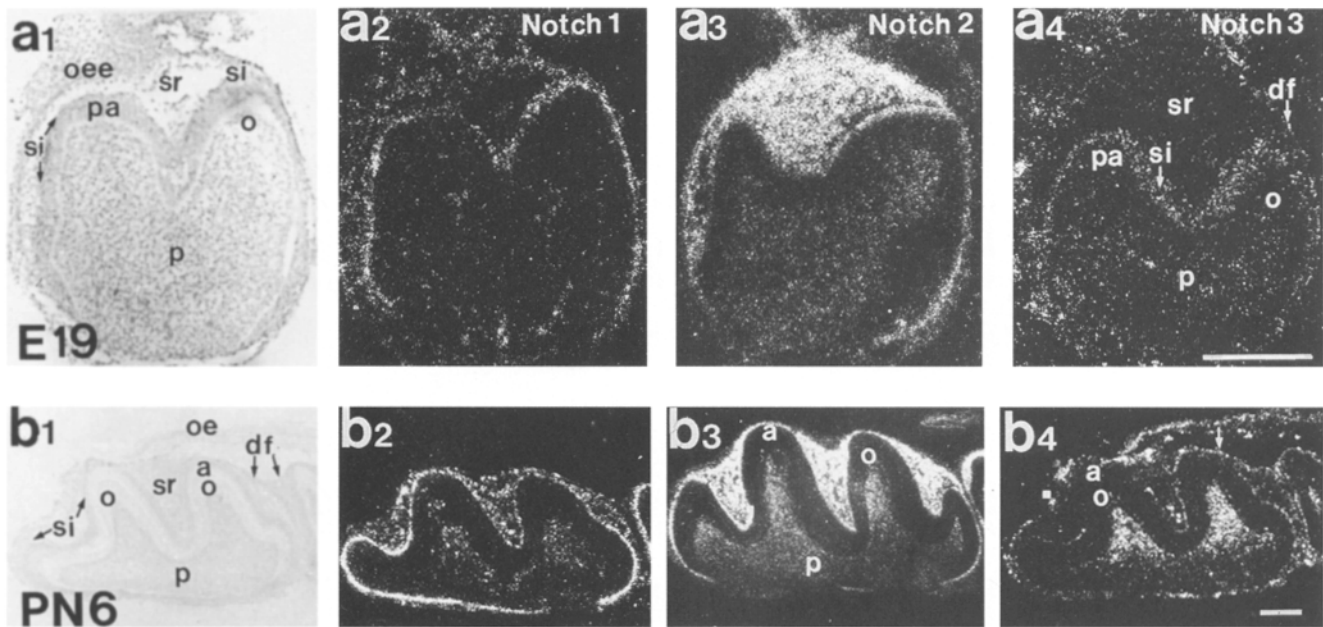


Figure 3. Localization of *Notch 1*, *2*, and *3* mRNAs by in situ hybridization in embryonic and postnatal mouse molar teeth (E19-PN6). (x1) Bright-field micrographs; (x2, x3, and x4) dark-field micrographs. (a) At E19, transcripts for all *Notch* genes are detected in the enamel organ, but they are absent from preameloblasts (*pa*). *Notch 1* and *3* mRNA are mainly expressed in stratum intermedium (*si*), whereas *Notch 2* mRNA is also found in stellate reticulum (*sr*) and outer enamel epithelium (*oe*). A weak hybridization signal is observed in dental papilla mesenchyme (*p*), but is absent from polarizing odontoblasts (*o*). *Notch 3* transcripts are also found in dental follicular mesenchyme (*df*). (b) At PN6, the expression pattern of *Notch* genes persisted in the enamel organ. No hybridization signal is seen in functional ameloblasts (*a*). In dental papilla, transcripts for all three *Notch* genes are absent from functional odontoblasts (*o*), but the genes are strongly expressed in dental papilla mesenchyme of the cusp area. *Notch 3* transcripts are also found in dental follicular mesenchyme (*b4*, arrow). Abbreviation: *oe*, oral epithelium. Bars, 200 μ m.

Whole-mount In Situ Hybridization and Immunohistochemistry on Explants

For whole-mount in situ hybridization and immunohistochemistry the explants were fixed for 5 min in 100% methanol at -20°C , rehydrated with 75%, 50%, and 25% methanol in PBS, pH 7.4, washed with PBS and fixed overnight at 4°C with fresh 4% PFA. Whole-mount in situ hybridization was performed as previously described (Vainio et al., 1993; Mitsiadis et al., 1995a). The *Notch* riboprobes were labeled either with biotinylated UTP or digoxigenin (Boehringer Mannheim Corp., Indianapolis, IN). The probes were then diluted to 0.5 mg/ml in the hybridization mixture. Whole-mount immunohistochemistry was performed as described earlier (Mitsiadis et al., 1995a,b). The explants were incubated with the primary *Notch 1* or *Notch 2* antisera diluted 1:800 in PBS. Explants were then placed in 50% glycerol in PBS and observed under a stereomicroscope.

Tissue Recombination Experiments

The region of the molar tooth germ was carefully dissected from the rest of the lower jaw of E11-E16 mouse embryos. The distal parts of the developing hindlimb of E11 embryos were also dissected in Dulbecco's PBS. After dissection, tooth germs, oral and limb tissues were incubated 3 min in 2.25% trypsin and 0.75% pancreatin on ice, and then the epithelium was separated from the mesenchyme in DMEM supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD) under a stereomicroscope. Isolated dental and

oral epithelia were recombined with isolated dental mesenchyme of different embryonic ages on a polycarbonate membrane (pore size 0.1 μ m; Nuclepore Corp., Pleasanton, CA) supported by a metal grid (Trowell type). Similarly, limb mesenchyme was recombined to limb and dental epithelia of the same embryonic age (E11). The recombinants were cultured for 1-3 d in DMEM with 10% FCS in a humidified atmosphere of 5% CO_2 in air at 37°C . After culture the explants were fixed overnight in 4% PFA, dehydrated in ethanol and embedded in paraffin wax. Serial sections (5 μ m) were mounted on silanized slides, dried, and stored at 4°C . Some explants were used for analysis by whole mount immunohistochemistry.

Treatment and Implantation of Beads

Affi-gel blue agarose beads (100-200 mesh/75-150 μ m diameter; BioRad Labs., Hercules, CA) and anion exchange resin beads (AG 1-x2, 100-200 mesh/106-205 μ m diameter; BioRad Labs.) were used as carriers of FGF-2 and RA, respectively, as previously described (Vainio et al., 1993; Mitsiadis et al., 1995a). Recombinant FGF-2 protein (Boehringer Mannheim Corp.) was diluted with PBS, pH 7.4, to concentrations 100-250 μ g/ml and incubated with beads (50 beads/5 μ l per tube) for 40 min at room temperature. RA was diluted with dimethylsulfoxide (DMSO; Merck) to concentrations 1-100 μ g/ml and incubated with beads (50 beads/500 μ l per tube) for 30 min at room temperature. Beads were washed for 15 min in culture medium and then placed on top of the explants. Control beads for FGF-2 were treated identically with 0.1% BSA in PBS, whereas DMSO

layer. The strongest signal for *Notch 1* is observed in cells overlying the basal layer. *Notch 1* transcripts are also detected in dental papilla mesenchyme (*p*). *Notch 3* transcripts are detected only in vascular structures (arrow). (d) At E16 (early bell stage), all three *Notch* genes are expressed in the enamel organ, but the signal is absent from the basal layer of cells. The *Notch 1* signal is stronger in epithelial cells of the cervical loop area (arrows), whereas the *Notch 3* signal in cells overlying the basal layer. *Notch 2* and *3* transcripts are also observed in dental papilla. (e) At E17, *Notch 1* and *3* mRNAs are found in stratum intermedium (*si*), whereas *Notch 2* transcripts are detected in stratum intermedium, stellate reticulum (*sr*), and outer enamel epithelium (*oe*). A hybridization signal for the *Notch 2* and *3* genes is also detected in dental papilla. Abbreviations: *iee*, inner enamel epithelium; *m*, mandibular mesenchyme. Bars, 100 μ m.

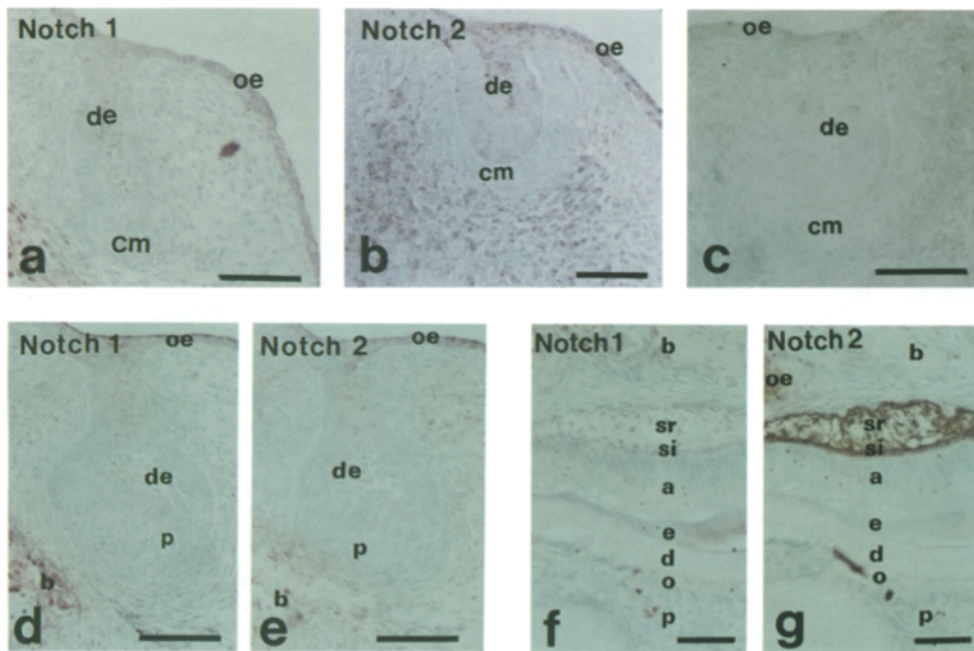


Figure 4. Immunolocalization of Notch 1 and 2 proteins in the developing first molar of the mouse. (a, d, and f) Notch 1 immunodetection; (b, e, and g) Notch 2 immunodetection. (a and b) During the bud stage (E13), Notch 1 and 2 proteins are localized in oral (oe) and dental (de) epithelium. The proteins are absent from dental epithelial cells of the basal layer and condensed dental mesenchyme (cm). The surrounding nondental mesenchyme is stained only for Notch 2. (c) A control for Notch 2 antiserum specificity. The antiserum was adsorbed with fusion protein before immunohistochemistry to a section of a bud staged tooth. (d and e) At cap stage (E14-E15), the expression patterns of Notch 1 and 2 persisted in dental

epithelium, whereas staining for Notch is not found in dental papilla mesenchyme (p). Both proteins are detected in cells of the alveolar bone (b). (f and g) High magnification of the cusp region of a molar tooth germ at PN8. Notch 1 and 2 immunoreactivities are observed in cells forming the alveolar bone and in oral epithelium. Strong Notch 2 immunoreactivity is observed in stellate reticulum (sr) and stratum intermedium (si), whereas Notch 1 staining in these layers is very faint. The staining observed in enamel (e) represents nonspecific binding of antibodies. Abbreviations: a, ameloblasts; o, odontoblasts; d, dentin. Bars, 100 μ m.

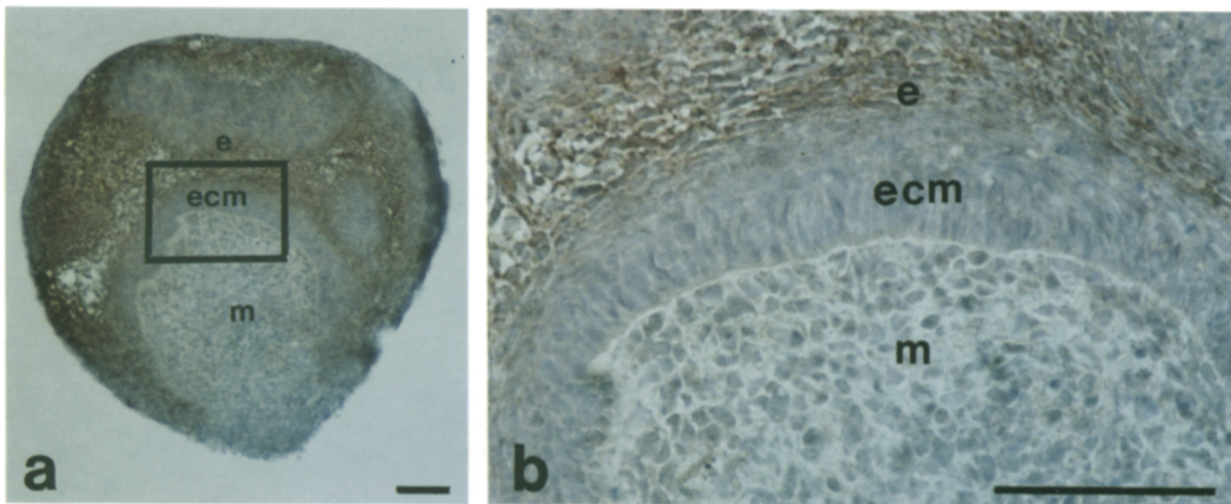


Figure 6. Immunolocalization of Notch 2 in explants of recombined E16 dental epithelium and E12 mandibular mesenchyme from the tooth region after 24 h of culture. (a) Notch 2 immunoreactivity is found in epithelial cells, except in cells contacting the mesenchyme (ecm). The reactivity is absent from mesenchymal cells. (b) Higher magnification of Fig. 6 a. Note that the immunoreactivity is localized on the surface of epithelial cells. Abbreviations: e, epithelium; m, mesenchyme. Bars, 50 μ m.

beads were used as control for RA. After 24 h of culture, the explants were analyzed by whole mount in situ hybridization or immunohistochemistry, as described above.

Results

The Expression Patterns of Notch Genes in the Developing Tooth Are Distinct and Correlate with Cell Fate Determination

The expression patterns of the *Notch 1*, *2*, and *3* genes in

the developing mouse tooth were analyzed by in situ hybridization. The various stages of tooth development are schematically illustrated in Fig. 1. By E11, expression of the *Notch* genes was observed in the superficial layer of the thickened dental epithelium (Fig. 2 a). Transcripts were absent from cells of the basal layer which interact with mesenchyme at this and during subsequent developmental stages. No hybridization signal was detected with sense probes at this or later developmental stages (data not shown).

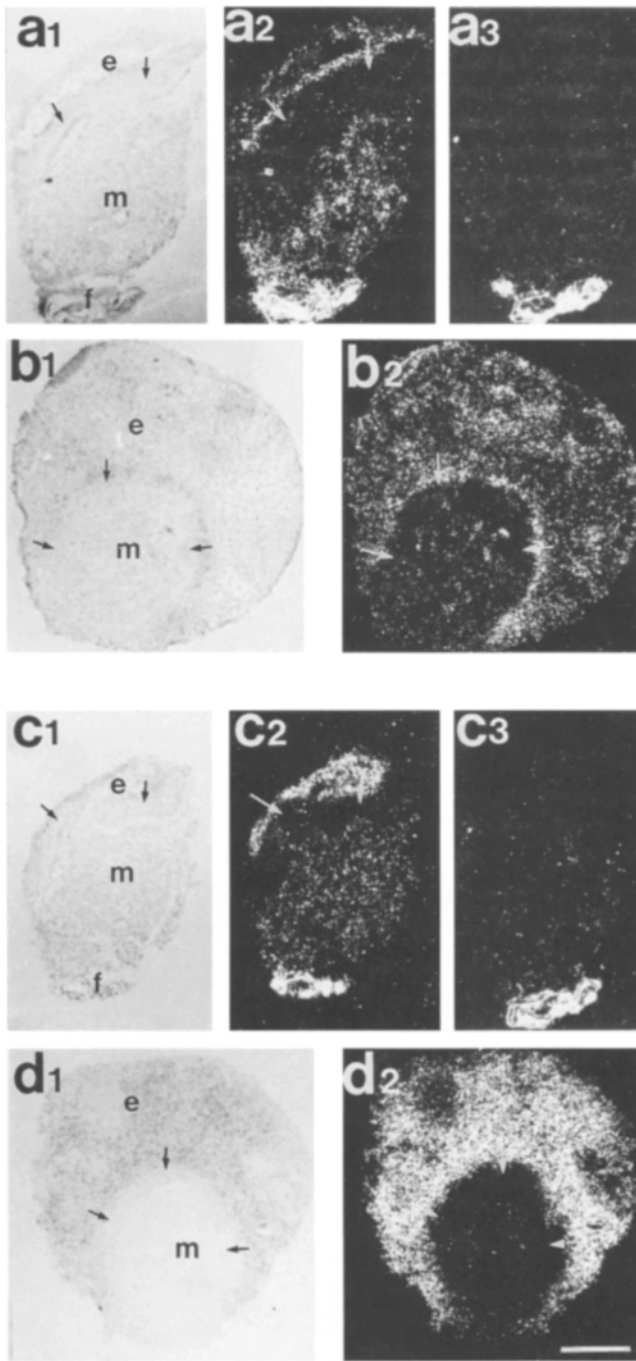


Figure 5. Localization of *Notch 1* and *2* transcripts by in situ hybridization in explants of recombined dental epithelium and mesenchyme after 24 h of culture. (*x1*) Bright-field micrographs; (*x2*, *x3*) dark-field micrographs. (*a*, *b*), *Notch 1* mRNA expression; (*c* and *d*) *Notch 2* mRNA expression. (*a3*, *c3*) Explants hybridized with sense *Notch 1* and *2* riboprobes. (*a* and *c*) Recombined E12 dental epithelium and E16 dental mesenchyme; (*b* and *d*) recombined E16 dental epithelium and E12 dental mesenchyme. (*a*) *Notch 1* transcripts are detected in both epithelial and mesenchymal cells, but are absent in epithelial cells contacting the mesenchyme (arrows). The signal found in filter (*f*) is an artifact. (*b*) *Notch 1* transcripts are found in the two recombined tissues, but the signal is absent in epithelial cells adjacent to mesenchyme (arrows). The strongest signal is observed in epithelial cells contacting the layer of nonexpressing *Notch 1* cells. (*c*) An intense *Notch 2* mRNA signal is found in epithelial cells, except in cells contacting

At E13 (bud stage), all three *Notch* genes were expressed in the superficial cell layers of dental epithelium, whereas the transcripts were absent from the condensed mesenchyme (Fig. 2 *b*). During the cap stage (E14-E15), *Notch 1* and *2* genes were expressed in dental epithelium, with the exception of its basal part, which will later differentiate into ameloblasts (Fig. 2 *c*). Only *Notch 1* transcripts were found in the dental mesenchyme (dental papilla) (Fig. 2 *c2*). Neither dental epithelium nor mesenchyme expressed the *Notch 3* gene (Fig. 2 *c4*).

By E16 the epithelium (enamel organ) acquires the bell configuration. During this stage (E16-E19) the expression patterns of the *Notch* genes became progressively restricted to specific subpopulations of cells of the enamel organ. The highest expression of *Notch 1* was seen in cells located in the cervical loop area and in stratum intermedium (Figs. 2, *d2* and *e2*, and 3 *a2*), while *Notch 2* transcripts were most abundant in stellate reticulum (Figs. 2, *d3* and *e3*, and 3 *a3*). *Notch 3* transcripts were detected only in cells of the stratum intermedium (Figs. 2, *d4* and *e4*, and 3 *a4*). Transcripts of all three genes were absent in preameloblasts. The *Notch 2* and *3* genes were expressed in the dental papilla mesenchyme (Figs. 2, *d4*, *e3*, and *e4*, and 3 *a3*), whereas the expression of *Notch 1* was correlated with endothelial cells (Figs. 2, *d2* and *e2*, and 3 *a2*).

During the terminal differentiation of mesenchymal cells into odontoblasts and of preameloblasts into ameloblasts (PN1-PN6), the expression patterns of the *Notch* genes in dental epithelium remained similar as during E17-E19 (Fig. 3 *b*). In dental papilla mesenchyme, all three *Notch* genes were transiently expressed in cells underlying differentiating odontoblasts (Fig. 3 *b*). We did not observe expression of any of the *Notch* genes in differentiated odontoblasts and ameloblasts.

The Distribution Patterns of *Notch 1* and *2* Proteins Correlate with mRNA Expression

To analyze Notch protein expression, we raised polyclonal antisera against portions of the Notch 1 and 2 extracellular domains. Immunoreactivities for Notch 1 and 2 were evident in dental epithelium during embryonic tooth development, but were absent from cells adjacent to the dental mesenchyme (Fig. 4, *a*, *b*, *d*, and *e*). Immunoreactivity was found in all cell layers of the oral epithelium (Fig. 4, *a* and *b*). The dental mesenchyme was negative for Notch 1 and 2. During advanced tooth morphogenesis (PN8), the two antisera produced distinct patterns: Notch 2 immunoreactivity was present in cells of the stellate reticulum, whereas these cells were negative for Notch 1 (Fig. 4, *f* and *g*). In control sections stained with antibodies which had first been incubated with the Notch 1 and 2 fusion proteins

the mesenchyme (arrows). Some transcripts are also detected in mesenchyme. The signal observed in filter *f* is nonspecific. (*b*) *Notch 2* transcripts are observed in all epithelial cells except in cells adjacent to mesenchyme (arrows). The signal is also absent from mesenchyme. Abbreviations: *e*, epithelium; *m*, mesenchyme. Bar, 100 μ m.

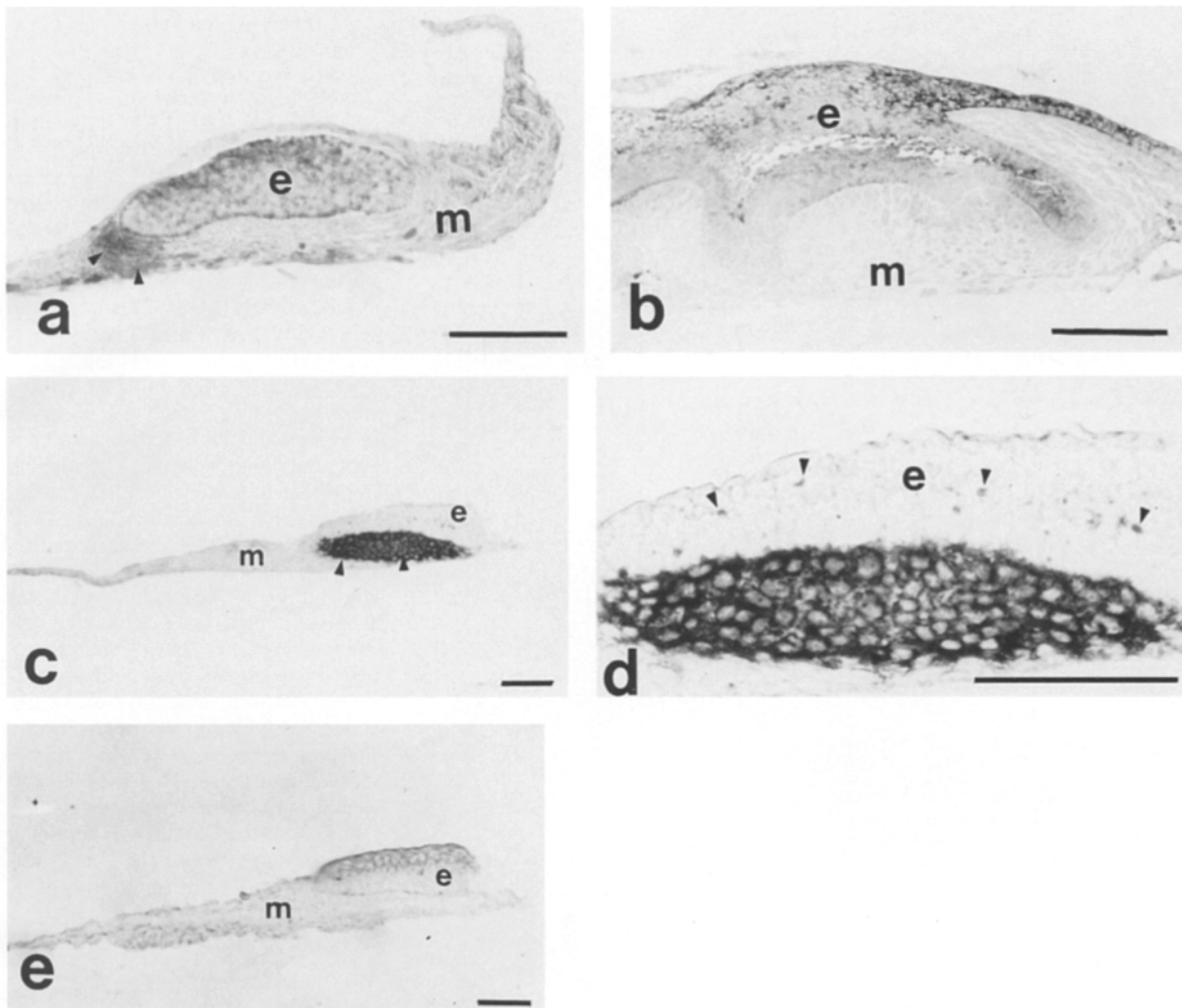


Figure 7. Immunolocalization of Notch 1 and 2 proteins in explants of recombined mandibular arch epithelium and mesenchyme from the tooth region (E11.5-E14). (a) Notch 2 immunoreactivity is localized on the surface of all epithelial cells and some mesenchymal cells adjacent to the epithelium (*arrowheads*) in the E11.5 recombinants cultured for 12 h. (b) Notch 2 immunoreactivity is found in epithelial cells, except in cells contacting the mesenchyme in the E14 recombinants cultured for 2 d. The reactivity is absent from mesenchymal cells. (c) Punctuated Notch 2 immunoreactivity is found in epithelial cells in the E12 recombinants cultured for 3 d. A strong immunoreactivity is observed in mesenchymal cells adjacent to epithelium (*arrowheads*). (d) Higher magnification of Fig. 7 c. Note the punctuated staining in epithelium (*arrowheads*). (e) Notch 1 immunoreactivity is found in epithelial cells, except in cells contacting the mesenchyme in the E12 recombinants cultured for 3 d. The staining is absent from mesenchymal cells. Abbreviations: *e*, epithelium; *m*, mesenchyme. Bars, 50 μ m.

used for immunization the immunoreactivities were eliminated (Fig. 4 c, and data not shown).

Expression of Notch mRNAs and Proteins in Developing Tooth Is Regulated by Interactions between Epithelium and Mesenchyme

The absence of *Notch* transcripts from the basal epithelial cells in close proximity to dental mesenchyme suggested that *Notch* expression may, in part, be regulated by tissue interactions. To address this possibility we examined *Notch* expression in explants of both homo- and heterotypic recombinants. *Notch* expression was analyzed by in situ hybridization and immunohistochemistry after 0.5–3 d in culture.

In homotypic recombinants from dental tissues cultured

for 24 h, *Notch* mRNA expression was not observed in epithelial cells in contact with mesenchyme (Fig. 5). This was the case in all epithelio-mesenchymal combinations tested, irrespective of the age of the tissues. For example, E12 dental epithelium cultured in close contact with E16 dental mesenchyme behaved similarly to E16 epithelium cultured with E12 mesenchyme in terms of suppression of *Notch* expression in epithelial cells (Fig. 5, a2–d2). Immunohistochemical analysis showed that Notch 1 and 2 staining was absent from epithelial cells close to the mesenchyme (Figs. 6 and 7 b). However, when recombinants were cultured for a shorter period (12 h) (Fig. 7 a), or when dental epithelial tissues were cultured alone (data not shown), the proteins were expressed in all epithelial cells. In the homotypic explant cultures, Notch 2, but not Notch 1, immunoreactivity was upregulated in the mesen-

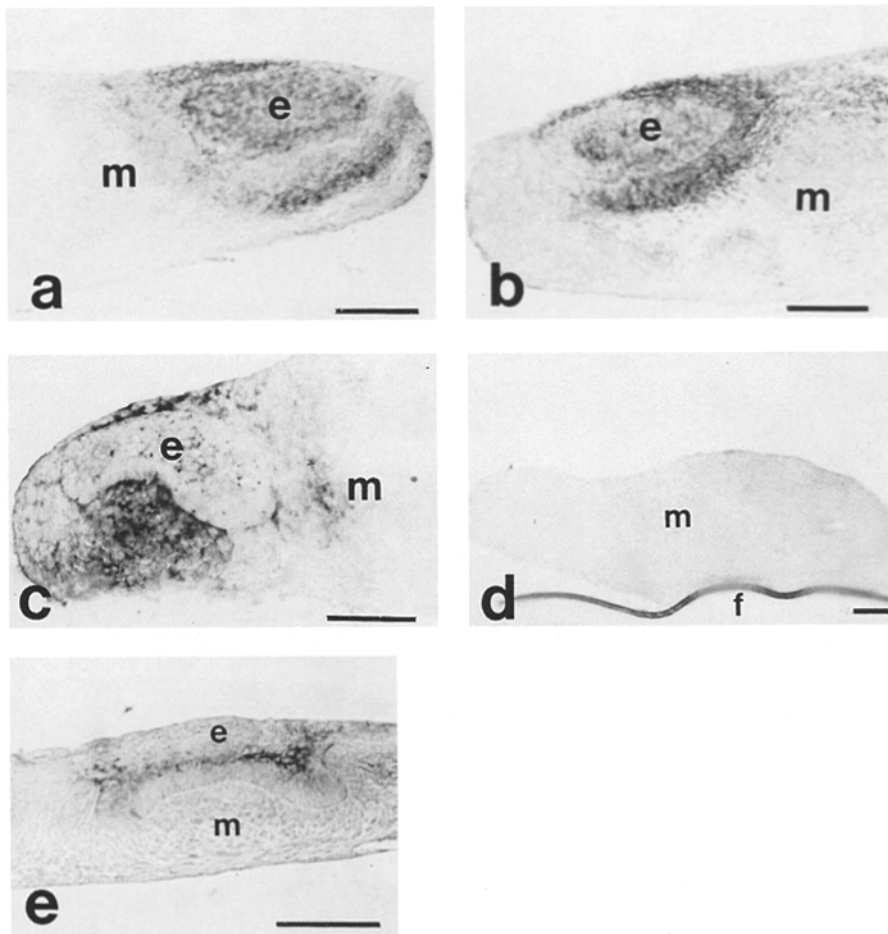


Figure 8. Immunolocalization of Notch 1 and 2 proteins in heterotypic recombinants. (a, b, and c) Mandibular arch epithelium from the tooth area (E11) and limb mesenchyme (E11). (d) E11 limb mesenchyme. (e) Oral epithelium from the diastemal region (E12.5) and dental mesenchyme (E12.5). The explants were cultured for 12 h (a), 24–32 h (b and c), and 3 d (e). (a) Notch 2 reactivity is found in all epithelial cells and in mesenchymal cells adjacent to the epithelium. (b) Notch 2 staining is observed in epithelial cells, except in cells contacting the mesenchyme. The staining is evident in mesenchymal cells adjacent to the epithelium. (c) Notch 2 immunoreactivity is observed in epithelium and in mesenchyme adjacent to the epithelium. The staining is absent from epithelial cells contacting the mesenchyme. (d) Notch 2 immunoreactivity is absent from limb mesenchyme cultured alone. (e) Notch 1 immunoreactivity is absent from the layer of epithelial cells which are in close contact with the recombined dental mesenchyme, whereas it is localized on the surface of epithelial cells overlying this layer of cells. Abbreviations: e, epithelium; m, mesenchyme; f, filter. Bars, 50 μ m.

chyme after 3 d in culture (Fig. 7, c–e).

To learn whether the regulatory mechanisms observed in the homotypic recombinants were specific to tooth, or more general, we next examined heterotypic recombinants. E11 limb mesenchyme, which was negative for Notch expression when cultured alone (Fig. 8 d), was recombined with E11 dental epithelium and cultured for 24–48 h. Intense Notch 1 and 2 staining was found in the limb mesenchyme adjacent to dental epithelium (Fig. 8, a–c), and the dental epithelial cells showed a downregulation of Notch 1 and 2 protein expression (Figs. 8, b and c, and data not shown). The negative regulation exerted by mesenchyme thus also seems to function in heterotypic recombinants.

Heterotypic and heterochronic tissue recombination studies have shown that the odontogenic potential resides in the presumptive dental epithelium until E12, and then shifts to the condensing mesenchyme (Kollar and Baird, 1969; Mina and Kollar, 1987). We asked then if dental mesenchyme could downregulate Notch expression in heterotypic explants with nondental epithelium. E12.5 dental mesenchyme was cultured for 3 d together with E12.5 nondental jaw epithelium in which all cells initially showed Notch 1 and 2 immunoreactivities (see Fig. 4, a and b). After culture, the epithelium had invaded the dental mesenchyme and acquired a cap-like configuration. Epithelial cells, except for those in close proximity to dental mesen-

chyme, were positive for Notch 1 and 2 (Fig. 8 e, and data not shown), indicating that dental mesenchyme had caused downregulation of Notch expression in basal epithelial cells. This expression pattern was similar to that in the tooth germ in vivo.

RA, but Not FGF-2, Induces Expression of Notch Genes in Dental Mesenchyme

While the early dental mesenchyme (E11–E12) does not express *Notch* genes at high levels (Fig. 2, a and b), expression was transiently upregulated at more advanced stages (Figs. 2, c–e and 3 b). This indicated the possibility that *Notch* expression in dental mesenchyme is activated by epithelium-derived signal molecules, such as retinoic acid (RA) and fibroblast growth factor-2 (FGF-2).

Resin beads loaded with different concentrations of RA, and agarose or heparin acrylic beads soaked in FGF-2, were implanted into dental mesenchyme from different developmental stages and *Notch* expression was analyzed after 16–24 h by whole-mount in situ hybridization and whole-mount immunohistochemistry. In E11–E12 tissue recombinants, beads soaked in 50–100 μ g/ml RA induced expression of mRNA from all three *Notch* genes in cells adjacent to the beads (Fig. 9), while lower RA concentrations (1–5 μ g/ml) had no apparent effect on *Notch* gene expression (data not shown). Similarly, RA beads (100 μ g/

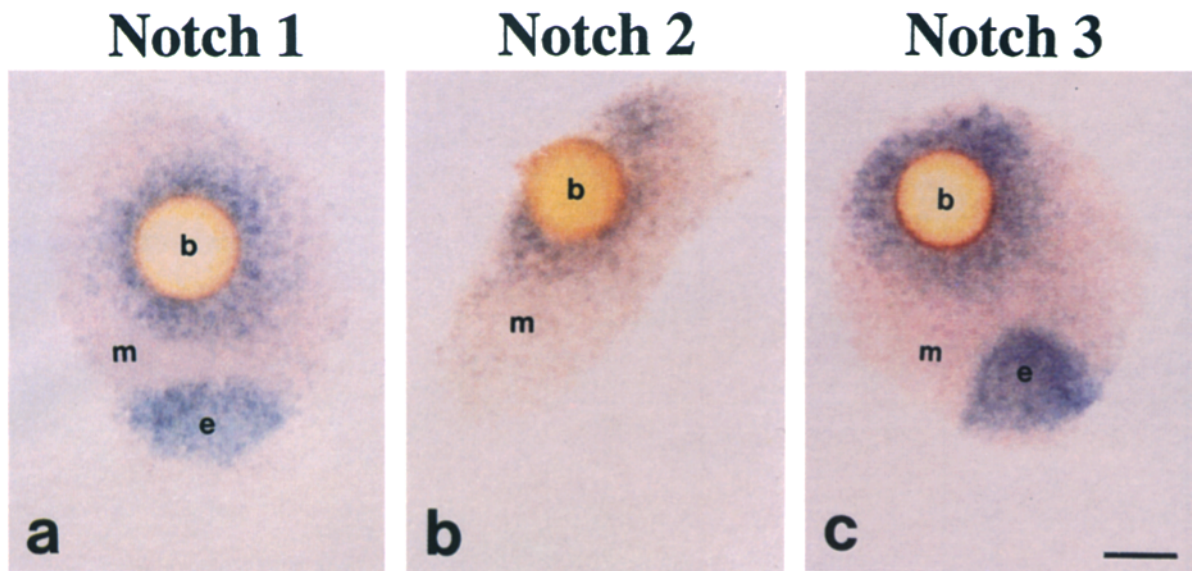


Figure 9. Stimulation of the *Notch 1*, *2*, and *3* gene expression in the mandibular mesenchyme from the tooth area by retinoic acid (RA). E11-E12 mesenchyme was cultured for 24 h with implanted beads soaked in RA (100 $\mu\text{g/ml}$). The mesenchyme was cultured either alone (*b*) or in contact with presumptive dental epithelium (*a* and *c*). The expression of the three *Notch* genes was monitored by whole mount in situ hybridization (*a*) *Notch 1*, (*b*) *Notch 2*, and (*c*) *Notch 3*. Expression of all three *Notch* genes is found in mesenchymal cells surrounding the RA releasing bead. The recombined epithelium is positive but has not induced *Notch* mRNA expression in the adjacent mesenchyme. Abbreviations: *e*, epithelium; *m*, mesenchyme; *b*, bead. Bar, 100 μm .

ml) evoked the expression of Notch 1 and 2 proteins in dental mesenchyme (data not shown). DMSO control beads had no effects on *Notch* expression (data not shown). In the E13-E14 dental mesenchyme, RA beads were unable to induce the expression of Notch 1 and 2 proteins, suggesting that the response to RA is dependent on the developmental stage. In a similar set of experiments we examined the response of mesenchyme to beads soaked in recombinant FGF-2 (100–250 $\mu\text{g/ml}$). We could not detect any Notch 1 or 2 immunoreactivity or mRNA expression in mesenchymal cells surrounding the FGF-2 beads (data not shown).

Discussion

Downregulation of Notch Expression Is Associated with Determination of Epithelial Cells into the Ameloblastic Lineage

In this report we show that the *Notch* genes are developmentally regulated in the rodent tooth. *Notch 1*, *2*, and *3* exhibit overlapping but distinct expression patterns during tooth morphogenesis, and their expression is downregulated after completion of odontogenesis (Fig. 10 *a*). *Notch* genes are important for cell fate decisions in *Drosophila* and vertebrates, but the exact mechanism of Notch action is not yet understood. It has been postulated that the role of Notch is to block differentiation by maintaining the competence of undifferentiated cells (Coffman et al., 1993; Fortini and Artavanis-Tsakonas, 1993). This implies that *Notch* expression would preferentially be found in cell types that are not terminally differentiated. Several expression patterns during tooth development can be viewed in this light. Odontoblasts, which are highly differentiated

mesenchymal cells, do not express detectable levels of *Notch* mRNA. In contrast, mesenchymal cells known to maintain their ability to later differentiate into odontoblasts upon specific signals, for example during reparative dentinogenesis, do express the *Notch* genes. In dental epithelium, the *Notch* expression patterns reveal a previously unknown prepatterning at very early stages, namely a subdivision of the E11 epithelium in *Notch* expressing and non-expressing regions. Thus, the nonexpressing region, located close to the dental mesenchyme, contains cells that later give rise to ameloblasts producing the enamel matrix, and may reflect an early determination step in this process. Amelogenin, which is the main structural component of enamel matrix, is expressed in the epithelial cells at cap stage (E14) (Couwenhoven and Snead, 1994). Hence, downregulation of *Notch* genes in these cells precedes the cell type specific gene expression by three days. It is thus possible that Notch signaling in epithelial cells prevents them from adopting the ameloblast fate.

Negative Regulation of Dental Epithelial Notch Expression by Mesenchyme

The downregulation of *Notch* expression in epithelial cells in close proximity to the dental papilla mesenchyme in vivo was reproduced in both homotypic and heterotypic recombination experiments in vitro (Fig. 10 *b*). A zone of Notch-negative epithelial cells was created adjacent to the recombined mesenchyme, while all epithelial cells expressed *Notch* when the epithelium was cultured alone. This suggests that the absence of *Notch* expression in the basal epithelial cells depends on negative regulation by mesenchymal cells. Limb mesenchyme also caused downregulation of Notch protein expression in dental epithe-

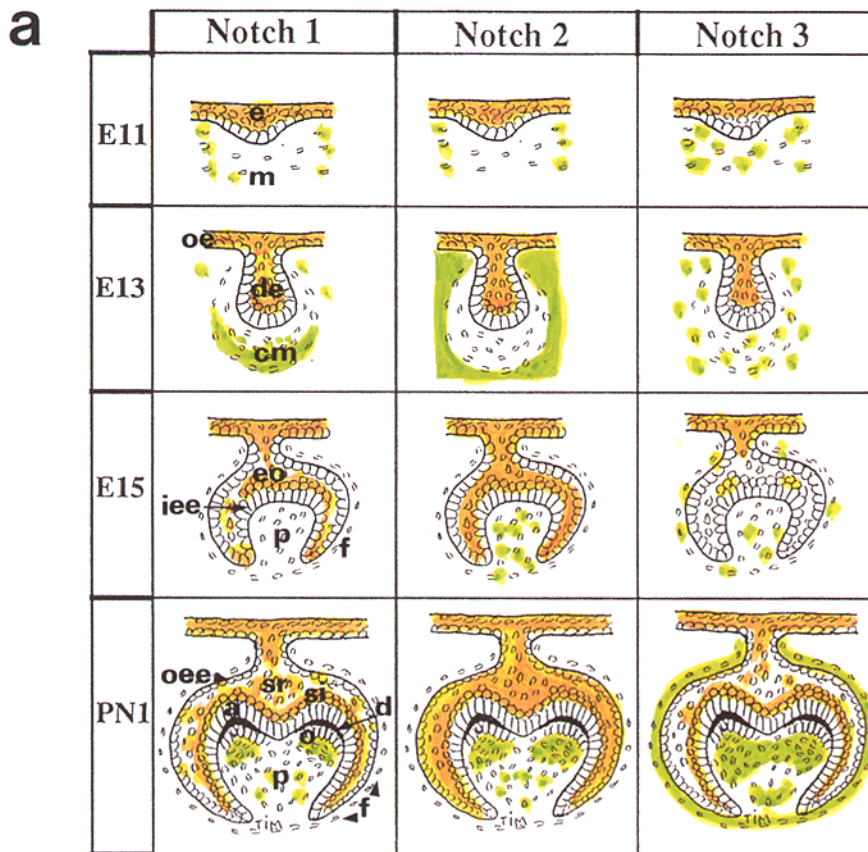
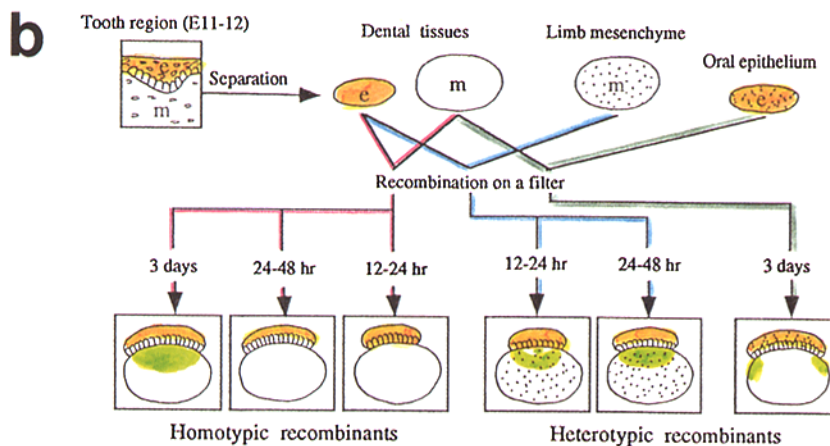


Figure 10. (a) Schematic illustration of the expression patterns of the *Notch* genes in the developing first molar of the mouse. The expression of the *Notch* genes in epithelium (*e*) is indicated in orange, and in mesenchyme (*m*) in green. (b) The design of the experiments used to analyze the regulation of the *Notch* genes and proteins by tissue interactions. Epithelia and mesenchymes from the tooth, oral area, and limb are separated and cultured either alone or as recombinants (homotypic = red lines, and heterotypic = green and blue lines). The *Notch* genes and proteins are expressed in all cells of the epithelia cultured alone (orange color), whereas they are absent from mesenchymes cultured alone. In recombinants, *Notch* expression is downregulated in the layer of epithelial cells contacting the mesenchyme after 24 h of culture, but never before. Furthermore, the dental epithelium induces *Notch* expression in the adjacent dental and limb mesenchyme (green color). Abbreviations: *de*, dental epithelium; *cm*, condensed mesenchyme; *oe*, oral epithelium; *f*, dental follicle; *eo*, enamel organ; *iee*, inner enamel epithelium; *p*, dental papilla mesenchyme; *sr*, stellate reticulum; *si*, stratum intermedium; *oee*, outer enamel epithelium; *a*, ameloblasts; *o*, odontoblasts; *d*, dentin; *E*, embryonic day; *PN*, postnatal day.



lium, indicating that the mesenchymal effect may not be specific to dental tissue. In an alternative model, the E11 dental epithelium could first “respecify” the limb mesenchyme, which in turn could signal back to epithelium affecting *Notch* expression. This hypothesis may receive support by the fact that the downregulation of *Notch* protein expression in epithelium is seen first after 24 h of culture.

The mechanism by which mesenchyme downregulates *Notch* expression in epithelium could involve either mesenchymal secreted factors acting over a short distance or

components of the basement membrane. A putative secreted factor may bind to the basement membrane separating the epithelium from the dental papilla mesenchyme, and exert local effects on cells contacting the basement membrane. This membrane is removed when epithelium and mesenchyme are separated by proteolytic enzymes, but is rapidly restored when epithelium and mesenchyme are cultured in recombination (Ruch, 1987). Both epithelium and mesenchyme contribute to the formation of the basement membrane and the restoration has been shown

to require both tissues. Several growth factors, such as MK, transforming growth factor beta (TGF β), FGFs, and platelet-derived growth factors (PDGFs), have been shown to bind to basement membranes (for review see Adams and Watt, 1993; Mitsiadis et al., 1995a,b). Downregulation of *Notch* in early dental epithelium and mesenchyme is associated with expression of several growth factors and extracellular matrix molecules involved at the epithelial-mesenchymal interface, such as MK (Mitsiadis et al., 1995a), BMP-2 and -4 (Vainio et al., 1993), syndecan-1, and tenascin (Vainio et al., 1989). Whether these or other molecules are involved in downregulation of *Notch* is being studied. The presence of FGF-2 does not generate *Notch* expression, and our preliminary findings indicate that this is the case also for BMPs. These results are consistent with the tissue recombination experiments: dental epithelium caused the induction of *Notch* expression in dental mesenchyme only after several days of culture, whereas a transient expression of syndecan-1, tenascin, and BMPs is observed after 16–24 h of culture (Vainio et al., 1989, 1993) which may downregulate *Notch* expression during that period. Taken together these findings indicate a complex interplay of negative and positive signaling to establish different levels of *Notch* expression in regions of epithelial-mesenchymal interactions, which may be important for cytodifferentiation.

Notch Genes as Possible Mediators of RA Effects

The specific upregulation of *Notch* expression by exposure to RA, but not to FGF-2, suggests that Notch activity may play a role in the cellular response to RA. RA is a powerful signaling molecule acting as a diffusible morphogen or as a local inducer (Tabin, 1991; Tickle, 1991). Exposure to exogenous RA during pregnancy leads to congenital malformations, including cleft palate and skeletal defects (Geelen, 1979). RA binds to a family of specific nuclear receptors (retinoic acid receptors RARs), which are transcription factors (Nagpal et al., 1993). RARs are expressed in the developing tooth of the mouse (Bloch-Zupan et al., 1994), and it has been shown that the excess of retinol, which converts to RA, alters the pattern of odontogenesis in vitro, by producing supernumerary tooth buds in the distal region of the mandible (Kronmiller et al., 1994). Teratogenic exposure to RA leads to altered expression of *Hox* genes, which are important developmental control genes (Kessel and Gruss, 1991; Marshall et al., 1992). The induction of *Notch* genes by RA is particularly interesting in this context, since removal of *Notch* genes (Swiatek et al., 1994) as well as constitutive Notch signaling (Ellisen et al., 1991; Jhappan et al., 1992) produce dramatic effects during development. It is therefore possible that the teratogenic effects seen after RA exposure may be, in part, caused by ectopic induction of *Notch* expression.

It is not yet known how *Notch* induction is mediated: it could be a direct effect of RARs on *Notch* promoters or require the action of Hox transcription factors. The distribution of the homeobox-containing genes *msx-1* and *msx-2* show striking correlations with tooth patterning (MacKenzie et al., 1991, 1992), and the deficient function of the *msx-1* gene in transgenic mice inhibits tooth development (Sato-kata and Maas, 1994). The expression of *msx-1* and *msx-2*

in early dental mesenchyme is regulated by epithelial-mesenchymal interactions (Jowett et al., 1993) and BMP-4 (Vainio et al., 1993), suggesting that these genes may also be involved in *Notch* downregulation.

In conclusion, our data provide the first insights into the regulation of vertebrate *Notch* genes by RA and epithelial-mesenchymal interactions. An involvement of these genes in the RA signaling cascade may further increase our understanding of both their expression patterns and the biological roles of Notch molecules during the embryonic development of vertebrates.

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