Timing of the expression of enamel gene products during mouse tooth development

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ABSTRACT In order to understand the mechanisms involved in tooth development it is important to define the timing for tissue-specific gene expression. A consequence of ameloblast cell differentiation is the sequential expression of tissue-specific genes whose products form the enamel extracellular matrix. The ameloblast phenotype has been characterized as consisting of two major classes of proteins: amelogenins and non-amelogenin proteins such as anionic enamel proteins (enamephins, tuftelin, sulfated proteins) and enamel proteases. The postulated functions for the anionic enamel proteins are as nucleators for hydroxyapatite crystal formation while amelogenins control the crystal size, growth and orientation. While the amelogenins have been well characterized, detailed knowledge for anionic enamel proteins has been sparse. In the present study, we designed experiments to characterize one of the anionic enamel proteins from mouse molars, tuftelin, and to determine the timing of expression of this protein during molar tooth development. Our results showed the initial detection of tuftelin transcripts within proliferating inner enamel epithelial cells at very early stages of tooth development (13 days of embryonic development equivalent to the bud stage of tooth development). These data provide direct evidence that invalidates previous dogmas that enamel proteins were synthesized by polarized, non-dividing, fully differentiated ameloblast cells. In addition, tuftelin was found to be synthesized also by dental papilla mesenchyme cells suggesting that this protein is not enamel-specific. These data taken together open the possibility that the tuftelin present in the dentino-enamel junction could be secreted by both, preodontoblast cells and preameloblast cells. It might also suggest a possible different role for tuftelin than nucleator of hydroxyapatite crystals.

KEY WORDS: tooth development, amelogenesis, tuftelin, amelogenin, ameloblastin, mouse

Introduction

Tooth development, like development of other epidermal organs (mammary gland, lung, kidney, hair, skin, feathers, etc.), is mediated by reciprocal interdependent epithelial-mesenchymal interactions resulting in the differentiation of mesenchymal cells into odontoblast cells and epithelial cells into ameloblast cells (for reviews see Thesleff and Hurmerinta, 1981; Kollar, 1983; Slavkin et al., 1984, 1988b; Ruch, 1965, 1988; Lumsden, 1987, 1988; Slavkin, 1990; Thesleff et al., 1990, 1991). A consequence of ameloblast cell differentiation is the sequential expression of tissue-specific genes whose products form the enamel extracellular matrix. This process, known as amelogenesis, has been divided into two major phases: i) secretory stage, where ameloblast cells synthesize and secrete tissue-specific proteins into the forming enamel extracellular matrix, and ii) maturation stage, where removal of organic components and water from the extracellular matrix is followed by formation of calcium hydroxyapatite crystals resulting in complete mineralization of the enamel extracellular matrix.

The ameloblast phenotype has been characterized as consisting of two major classes of proteins: i) hydrophobic proteins known as amelogenins (for a recent review see Fincham et al., 1992) and ii) non-amelogenin proteins such as anionic enamel proteins (enamephins, tuft proteins, tuftelin, ameloblastin, sulfated proteins) and enamel proteases (for a recent review see Zeichner-David et al., 1995). Amelogenins are the most abundant comprising approximately 90% of the enamel proteins secreted by the ameloblasts.
last cells (Termine et al., 1980; Fincham and Belcourt, 1985). Sequence comparisons of amelogenins obtained from different species show striking homology for these proteins indicating a high degree of conservation (Fincham et al., 1992). The postulated functions for amelogenins have ranged from calcium chelators (Glimcher, 1979), inhibitors of crystal growth (Doi et al., 1984; Aoba et al., 1987) to regulators of crystal growth and orientation (Fearnhead, 1979; Aoba et al., 1987, 1989; Robinson et al., 1989; Fincham et al., 1992). Studies by Diekwisch et al. (1993) using antisense oligonucleotides in vitro, and more recently studies in vivo by Lyngstadtaas et al. (1996) using ribozymes to arrest amelogenin mRNA translation, provide strong support for the hypothesis that amelogenins control orientation and size of enamel crystals.

In contrast to amelogenins, detailed knowledge for the non-amelogenin proteins has just begun to emerge. Major factors contributing to the lack of information concerning these proteins have been their under-representation, aggravated by the presence of serum proteins in the mineralizing enamel extracellular matrix, which have made the isolation and characterization of these proteins extremely difficult (Okamura, 1983; Menanteau et al., 1987; Zeichner-David et al., 1987; Limeback and Simic, 1989; Strawich and Glimcher, 1990; Strawich et al., 1993). Another factor has been their short life, as in the case of the sulfated enamel proteins, which after 1-2 h are completely destroyed (Smith et al., 1995). Using a molecular biology approach, Deutsch et al. (1991) isolated and characterized a bovine cDNA clone for one of the anionic enamel proteins: tuftelin. More recently, another anionic enamel protein cDNA clone, ameloblastin/amelinin, was simultaneously characterized by two different groups of investigators using rat tooth cDNA libraries (Cerny et al., 1996; Krebsbach et al., 1996). The role of these proteins in amelogenesis remains unknown.

The developing mouse mandibular molar has been an excellent organ model to study the timing and positional information associated with the tissue-specific expression of molecular determinants involved in tooth development (for review see Slavkin et al., 1988b; Slavkin, 1990). A sequence of events associated with the development of the first mandibular molar (M1) beginning with neural crest cell migration, expression of cell-cell and cell substrate matrix adhesion molecules (CAMs and SAMs) at embryonic days 8.5-10 (E8.5-E10), formation of the dental lamina at E12, odontoblast and ameloblast cell differentiation, crown formation, root formation and tooth eruption at 28 days postnatal age has been described by Slavkin (1990). In this study, we wanted to determine the timing of expression for tuftelin. Experiments were designed to first characterize the mouse tuftelin, to prepare specific probes and to use these probes to determine the expression and localization of tuftelin during mouse molar tooth organogenesis at different stages of embryonic development.

**Results**

**Characterization of mouse tuftelin mRNA**

The only sequence available for tuftelin is derived from bovine teeth. Since this study utilizes developing mice teeth, and there could be species differences, we first needed to characterize the mouse tuftelin mRNA. Several combinations of PCR primers for bovine tuftelin were synthesized and tested for their ability to amplify a DNA fragment using cDNA prepared from mice teeth mRNA. From all the different combinations tested, only primers directed towards the coding sequence of the bovine tuftelin cDNA (257s-1000as or 38s-1000as) were capable of amplifying a DNA fragment of 734 bp and 950 bp respectively (Fig. 1). The PCR products were subcloned and the sequence of one of these clones, mT57, is shown in Figure 2. This clone represents approximately 1/3 of the bovine tuftelin mRNA, which is 2663 bp, containing approximately 75% of the coding sequence as shown in the diagram presented in Figure 3. Comparison of the mouse tuftelin deduced protein sequence with the bovine tuftelin protein deduced sequence (Fig. 4) indicated an 87% homology between both molecules.

**Expression of tuftelin mRNA during mouse embryonic and fetal development**

The timing of tuftelin mRNA expression during mouse embryonic and fetal development was determined using RT-PCR with mRNA extracted from Swiss Webster mouse mandibles obtained from 10 days gestation (E10) through newborn postnatal animals. Integrity of the mRNAs used in these experiments was tested using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (180bp). Tuftelin transcripts (743bp) were first determined in E13 mouse embryos (Fig. 5), with transcription continuing throughout postnatal stages. The presence of a doublet band suggests the possibility of alternative splicing of the tuftelin mRNA. Under comparable experimental conditions, amelogenin transcripts...
transcripts were first detected at E15. No tuftelin or amelogenin transcripts were detected when mRNA was obtained from other mouse tissues (heart or liver) and used as templates for RT-PCR. Non-specific bands could be seen in both, the tuftelin and amelogenin gels. These bands disappear when higher annealing temperatures were used.

Immunolocalization of tuftelin protein in developing mouse molars

To determine the presence of the tuftelin protein in the developing mouse molar an anti-peptide antibody against the bovine tuftelin sequence QSKDITIQELKKEIA was produced in rabbits. The specificity of the antibody was first characterized by Western blot immunostaining using 5 day-old mouse tooth enamel proteins

Fig. 2. DNA and deduced protein sequence of mouse tuftelin clone mTs7

(270bp) were first detected at E15. No tuftelin or amelogenin transcripts were detected when mRNA was obtained from other mouse tissues (heart or liver) and used as templates for RT-PCR. Non-specific bands could be seen in both, the tuftelin and amelogenin gels. These bands disappear when higher annealing temperatures were used.
Bovine coding sequence
PCR Primers
Mouse PCR amplified sequence
Bovine mRNA

Fig. 3. Map of bovine tuftelin. Representation of mouse clone mT57 in relation to the bovine tuftelin cDNA reported by Deutsch et al. (1991).

obtained by sequential extraction with acetic acid followed by GuHCl-EDTA (Fig. 6). Several Coomassie blue stained bands were present in both extracts; however, the antibody only cross-reacted with three bands present in the acetic acid extract. These proteins have molecular weights of approximately 70, 40 and 14 kDa. These data also indicate that most of the tuftelin(s) were extracted in the acetic acid solution (only a faint band of approximately 70 kDa remained in the GuHCl-EDTA extract). The tuftelin antibody does not cross-react with amelogenin as tested by the lack of immunostaining when a recombinant amelogenin (M179) (Simmer et al., 1994) was used in a western blot (data not shown).

To determine where in the tooth tuftelin is localized, immunohistochemical studies were conducted. At the light microscope level, tuftelin was detected mainly into the dentino-enamel junction (DEJ) as shown in Figure 7A. Discrete clusters of ameloblasts were actively secreting tuftelin which then appeared to diffuse within the enamel extracellular matrix (Fig. 7B). No staining in the dentin matrix was found. Comparison of tuftelin with amelogenin immunolocalization showed a striking difference between these proteins; amelogenins were intensely stained and found to be uniformly distributed across the forming enamel layer (Fig. 7C).

The presence of tuftelin in the ameloblast cells can be better seen by using transmission electron microscopy (Fig. 8A and B). Tuftelin can be seen in the ameloblast endoplasmic reticulum (Fig. 8C) as well as in the forming enamel extracellular matrix (Fig. 8B). Decalcification of the tissue did not affect the localization of tuftelin in the ameloblasts and in the forming enamel (Fig. 8D).

The presence of the tuftelin protein in mouse mandibular molars at different developmental stages was determined using immunohistochemistry with the tuftelin anti-peptide antibody. Tuftelin was first detected at E17 (Fig. 9A) where it appears to be synthesized and secreted to the extracellular matrix by pre-ameloblast cells. Some immunostaining was also detected in the dental papilla mesenchyme cells (DPM). At this stage of molar development amelogenin proteins were not detected (data not shown). Pre-ameloblast cells which contain immunoreactive tuftelin also showed positive immunostaining with an antibody against cyclin A (Fig. 10A) indicating that the cells are still participating in the cell cycle. These cells became immuno-negative for cyclin A when they polarized at E19 (Fig. 10B) with the cessation of the cell division. At this stage, cells were immunopositive for amelogenin (Fig. 10C).

Since the tuftelin antibody showed immunostaining in the DPM, we wanted to determine if this was due to non-specific cross-reactivity of the antibody with a component of these cells or it was indeed the cells synthesizing tuftelin. Poly(A)-containing RNA was prepared from a recently established DPM-derived cell line obtained from immortomouse (Zeichner-David et al., in preparation) and subjected to RT-PCR using tuftelin primers. The results shown in Figure 11 indicated that DPM cells also express tuftelin. The presence of a single band for tuftelin in this figure, as opposed to a doublet seen in Figure 5, might suggest that DPM cells only express one of the alternative spliced products. The DPM cells do not contain ameloblast cells, do not express amelogenin transcripts and are positive for dentin matrix proteins (George et al., 1993) as determined by RT-PCR (data not shown). These cells produce a dentin-like mineralized extracellular matrix in vitro (Zeichner-David et al., in preparation).
Discussion

The present study has defined the developmental expression of the anionic enamel protein tuftelin during mouse tooth development, which is summarized in Figure 12. Several surprising findings were encountered in this study: 1) detection of tuftelin transcripts at very early stages of tooth development (E13 or bud stage); 2) tuftelin being synthesized and secreted by non-polarized still dividing pre-ameloblast cells and 3) tuftelin being synthesized by DPM cells.

Morphological (Cohn, 1957; Slavkin et al., 1976) and biochemical (Slavkin et al., 1988a) studies describing mouse molar development have been reported. Until recently, it was assumed that transcription of enamel proteins was restricted to inner enamel epithelial cells that achieved terminal differentiation; in other words, cells that were polarized, elongated and withdrawn from the cell cycle (Snead et al., 1984, 1987, 1988; Ahmad and Ruch, 1987; Amar et al., 1989). Several years ago Snead et al. (1984, 1988) reported the initial expression of amelogenin at newborn stages of mouse molar tooth development (within non-dividing, polarized ameloblasts) as determined by cytoplasmic dot blot hybridization and in situ hybridization. With the advent of more sensitive mRNA phenotyping, such as RT-PCR techniques (PCR is as about 100-fold more sensitive than in situ hybridization and about 10,000 more sensitive than Northern blots or nuclease protection assays), the initial expression of amelogenin transcripts in the mouse molar were determined at E15 (cap stage of tooth development) within progenitor inner enamel epithelial cells as shown in this and other recent studies (Couwenhoven and Snead, 1994; Nakamura et al., 1994). Antibodies against non-defined anionic enamel proteins were used by Slavkin et al. (1988a) to demonstrate the initial synthesis of anionic proteins in E18 mouse first molars. However, no specific protein has been associated with these antibodies.

Using primers designed from the bovine tuftelin cDNA sequence (Deutsch et al., 1991), we were able to amplify a 950bp fragment of the mouse tuftelin cDNA. The deduced protein sequence of this fragment showed 87% homology to the deduced coding sequence of the bovine tuftelin (at present, no amino acid sequence of the actual tuftelin protein has been determined). These results indicate that tuftelin is highly conserved amongst species. Less homology exists between the mouse and bovine mRNAs at the 3'-end of the molecule as suggested by failure of primers directed to this region of the molecule to amplify the cDNA.

To gain more information concerning the tuftelin protein itself, an anti-peptide antibody was constructed. We used a peptide sequence previously demonstrated by Deutsch et al. (1991) to be antigenic. This antibody recognized three protein bands in a Western assay, thus suggesting that there are either (i) different tuftelin proteins which originated from different genes sharing a common epitope; (ii) different alternative spliced products derived from one gene; or (iii) multiple degradation products derived from...
one precursor protein by post-translational enzymatic processing. Additional biosynthetic studies will be necessary to discriminate between these three possibilities. An interesting observation from these experiments was the fact that the protein bands detected by the antibody were not stained by Coomassie blue. This can be interpreted as tuftelin being present in a very low concentration in the extract, not enough to be detected by the stain but sufficient to be detected by the antibody. The low representation of tuftelin relative to amelogenin was also observed by immunohistochemistry and immunocytochemistry.

The timing of expression and localization of tuftelin by immunodetection confirmed previous suggestions that enamel proteins are sequentially expressed with anionic proteins being synthesized prior to amelogenins and deposited along the dentino-enamel junction (DEJ) (Robinson et al., 1977; Deutsch et al., 1984; Slavkin et al., 1988a; Deutsch, 1989). Based on these observations and by analogy to the role of anionic proteins in biomineralization established in other systems (Lowenstam and Weiner, 1989) it has been suggested that the function of these anionic proteins is to serve as nucleators for calcium hydroxyapatite formation. Our studies showed that tuftelin continues to be expressed, although not uniformly but rather in ameloblast clusters, even when a thick layer of mineralizing enamel is formed (see Fig. 7). Thereafter, tuftelin immunostaining appears as diffusing into the mineralized enamel. If the tuftelin function is a nucleator of crystal formation, our observations suggest that once the initial nucleation takes place, tuftelin continues to either nucleate new crystals sites within the forming enamel, or regulates accretion to already existing crystals for extended growth. The immunocytochemical results do not support or reject these
possibilities. These studies only showed a higher proportion of amelogenins as compared to enamelines and were similar to studies reported by Herold et al. (1990) and Hayashi et al. (1986) using monoclonal antibodies against non defined enamelines.

The presence of tuftelin protein as determined by immunohistochemistry was not apparent until E17 (cap stage). The discrepancy between transcript detection and translation product detection might represent a difference in method sensitivity; PCR is extremely sensitive, whereas immunodetection requires a larger number of molecules for detection. However, another possible interpretation is that the earlier stages of transcription represent a physiological stage of ameloblast protodifferentiation, where very low levels of transcripts are expressed as suggested by Couwenhoven and Snead (1994). At the cap stage of tooth development, the basal lamina is still present, the inner enamel epithelial cells are not polarized and the cells are still dividing as demonstrated by the presence of cyclin A in the ameloblast cells; cyclin A is only present in the transition of G1 to S phase during the cell cycle (Carbonaro-Hall et al., 1993). Cyclin A was no longer detected once ameloblasts were polarized and actively secreting amelogenins (E19/newborn stages).

Using immunological methods for amelogenin, these proteins were also only evident at the bell stage (E19/newborn) in mouse neonatal molars (Snead et al., 1987; Slavkin et al., 1988a; Nakamura et al., 1994). In previous studies, we demonstrated the expression of a 46 kDa anionic protein cross-reactive with polyclonal antibodies against enamel proteins in E18 mouse embryos (bell stage) (Slavkin et al., 1988a) This protein was synthesized and secreted into the extracellular matrix (ECM) by inner enamel epithelial cells associated with an intact basal lamina, defined by Kallenbach's differentiation stages III and IV (1971, 1974). At E19, a second anionic protein (72 kDa) was detected in differentiation zones III-V. This stage included the initiation of mineralization as determined by the appearance of calcium hydroxyapatite crystals, electron-diffraction patterns and von Kossa staining for calcium-salt deposition. Newborn molar stages were associated with the continued expression of amelogenins by ameloblast (Kallenbach differentiation zone VI, secretory ameloblast with Tome's processes).

Couwenhoven and Snead (1994) recently showed that if the enamel organ epithelium was isolated and placed in culture, it required the presence of a reconstituted basement membrane gel (Matrigel) to express amelogenin transcripts. When these investigators placed isolated epithelia derived from dental lamina (E12), bud stage (E13) or early cap stage (E14) in culture, only cap stage-derived enamel organ epithelium expressed amelogenin in prolonged tissue culture; earlier stages of odontogenic epithelia did not express amelogenin transcripts. These experiments suggest that the instructive signal which controls amelogenin transcription occurs prior to or during early cap stage (Couwenhoven and Snead, 1994). Furthermore, these results also indicate that tuftelin and amelogenins have different transcription inducers,
Tissue preparation
Mandibles were obtained from Swiss Webster mice at different developmental stages ranging from E10-day pregnant mice (vaginal plug = day 0) through newborn (NB) mice. Tissues used for RNA extraction were dissected, immediately frozen in dry ice and kept at -80°C until used. Tissues for immunohistochemistry were fixed immediately in Carnoy's fixative (60% ethanol, 30% chloroform and 10% acetic acid) overnight at 4°C and then processed for paraffin embedding.

mRNA extraction
Poly(A) RNA was extracted from mouse mandibles using the Microfasttrack method (Invitrogen, San Diego CA) following the directions of the manufacturers. Briefly, the tissue is homogenized in the presence of RNase inhibitors and proteases, the DNA is sheared and the poly(A) RNA is separated from the rest of the RNAs by binding to oligo(T)-cellulose. RNA concentration is determined by A260nm (1 O.D. 260 = 40 µg RNA)

Fig. 9. Expression of tuftelin during mouse molar development. (A and B) An E17 mandible incubated with a 1:40 dilution of the tuftelin antibody (A) or no primary antibody (B). DPM, dental papilla mesenchyme; ECM, extracellular matrix; OEE, outer enamel epithelia; pAM, pre-ameloblast cells; pOd, pre-odontoblast cells.

since tuftelin and amelogenin are sequentially expressed, tuftelin being expressed at the bud stage (E13). These results support the hypothesis that multiple, sequential regulatory signals provided by the dental papilla mesenchyme control the biochemical differentiation of inner enamel epithelia into ameloblasts (Thesleff and Hurmerinta, 1981; Ruch, 1985, 1988; Lumsden, 1987, 1988; Thesleff et al., 1991).

It is clear from our studies that we are far away from understanding the role of these proteins in tooth development. The expression of tuftelin as early as the bud stage (Fig. 12), approximately 6 days before the onset of mineralization, which takes place at E19/ newborn stages in mouse molar tooth development (Slavkin et al., 1988a), questions the role of this protein in mineralization. Furthermore, the expression of this protein by DPM cells questions the term enamel-specific proteins. A similar situation has been shown for the dentin-specific protein dentin-sialoprotein (DSP, Ritchie et al., 1994). Using in situ hybridization, it was shown that although expressed mainly by odontoblast cells, pre-ameloblast cells express this protein too and then it is downregulated when the cells become ameloblasts (Ritchie et al., 1996). In situ hybridization studies using tuftelin probes will be necessary to determine if the reciprocal is true for tuftelin. These data might suggest that the initial deposition of tuftelin in the DEJ could come from the pre-odontoblast cells or could be a mixture of both, pre-odontoblast and pre-ameloblast secretion. Perhaps tuftelin is the "glue" that keeps the enamel and dentin layers attached to each other. If this is true, one can also speculate that the tuftelin gene is the gene responsible for some of the autosomal inherited cases of hypomaturation Amelogenesis Imperfecta where the enamel is particularly susceptible to chipping away from the underlying dentin matrix (Winter and Brook, 1975; Witkop and Sauk, 1976).

On the other hand, the possibility of a dual role for these proteins, one which takes place at early stages of tooth development (adhesion? signal transduction? induction? calcium transport? etc.) and a second role as nucleators of hydroxyapatite crystal formation at later stages of enamel formation can not yet be ruled out.

Materials and Methods

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Fig. 10. Expression of cyclin A during tooth development. Mouse mandibular molars incubated with anti-cyclin A antibody at E17 (A) and newborn (B) stages of development. (C) Represents newborn molar incubated with an amelogenin antibody. DPM, dental papilla mesenchyme; Am, ameloblast cells; pAm, pre-ameloblast cells.

Reverse transcription and PCR amplification of mRNA (RT-PCR)

The mRNAs were converted to cDNAs using the cDNA Cycle Kit from Invitrogen (San Diego, CA, USA) following the directions of the manufacturer. Briefly, 1 µg of poly(A) RNA obtained at each developmental stage was denatured with methyl mercury for 5 min at room temperature followed by the addition of β-mercaptoethanol and annealing with oligo dT primer.

RNAse inhibitor, dNTP, 5x Reaction Buffer and reverse transcriptase are added, the reaction mixture is incubated at 42°C for 1 h, boiled for 10 min, cooled in ice and 5 more units of reverse transcriptase are added. The reaction is incubated for one more hour at 42°C, boiled and cooled. The cDNA produced is stored at -80°C until ready to be used.

Synthetic oligonucleotides containing unique sequences characteristic for bovine tuftelin [5′= CGTAACTGGTGTACCTTG, 25′= TAAGTGTTACTTGAAAGG and 100′= GTGGCAATCTTCCTTGGAGC (Deutsch et al., 1991)], mouse amelogenin [5′= AACCAATGATGCCCCCTGC and 3′= CGTTAACCCTTCCTCCCGG] or rat glycerol phosphate dehydrogenase [5′= TGACATCAAGAAGGTGAAG and 3′= TCCCTGGAGGCGTGTAGGCC] were used for RT-PCR assays. The cDNA were amplified with Taq-polymerase (Perkin Elmer Cetus) in 50 µl reactions for 30 cycles (denaturation at 94°C, annealing of primer and fragment at 5°C less than the Tm, and primer extension at 72°C) using a Perkin Elmer Thermal Cycler. An aliquot of 20 µl of the reaction mixture was removed for agarose gel electrophoresis characterization. Gels were photographed after staining with ethidium bromide. Confirmation of the identity of the PCR product was obtained by direct DNA sequencing.

DNA sequencing

PCR products were fractionated in 2% agarose gels, bands were cut and the DNA recovered using GENECLEAN (BIO 101, San Diego, CA, USA). The purified DNA was either sequenced directly using the CircumVent Thermal Cycle Dideoxy DNA Sequencing kit (New England Biolabs) to obtain partial sequence or subcloned into the pcRT™IV vector for TA-cloning (Invitrogen, San Diego, CA, USA) to obtain the full sequence. Epicurean Coli Sure™ competent cells (Stratagene, La Jolla, CA, USA) were transformed and plated in LB agarose containing Ampicillin in the presence of X-GAL to produce white and blue colonies. White colonies were scraped, replated and the presence of the insert was confirmed by scraping the

Fig. 11. Expression of tuftelin by dental papilla mesenchyme cells. Tuftelin primers were used to amplify liver mRNA (2), whole mouse molar mRNA obtained from newborn mouse (3) or DPM cells mRNA (4). All RT-PCR reactions were initiated with 1 µg of poly(A)-RNA extracted from the respective tissues. The same gel is also shown embossed to enhance visualization of the bands.
Preparation and characterization of enamel protein antibodies

The antibody against tuftelin was prepared in rabbits as an antipeptide-antibody to the tuftelin deduced protein sequence QSKDTTIGELKEKIA (Research Genetics, Huntsville, AL, USA). An IgG fraction was prepared using a Protein A Affinity Pak column (Pierce, Rockford, IL, USA) following the procedures recommended by the manufacturers. The amelogenin antibody was a rabbit polyclonal antibody prepared against a recombinant mouse amelogenin (M179) expressed in E.coli (Simmer et al., 1994). The cyclin A antibody was a generous gift from Dr. Fred Hall (Children’s Hospital, Los Angeles, CA, USA).

Western immunoblot

Mouse enamel proteins were extracted sequentially in 0.5 M acetic acid followed by extraction of the residues in 4 M GuHCl-0.5 M EDTA. The samples were dialyzed and lyophilized. Aliquots of the extracts containing 50 μg of protein were fractionated in 12% polyacrylamide SDS gels and transferred to nitrocellulose. The filter was rinsed in TBS (50 mM Tris-HCl pH 8.0, 150 mM NaCl), free sites blocked by incubation in TBS containing 3% gelatin for 1 h and then incubated overnight with a 1:500 dilution of the tuftelin antipeptide-antibody in TBS containing 1% gelatin at room temperature with gentle shaking. The filter was rinsed and incubated with a goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad, Richmond, CA, USA) at a 1:3000 dilution for 1 h. After several washes with TBS the filter was immersed in the color development solution (0.3 mg/ml of NBT and 0.15 mg/ml BCIP in 0.1 M NaHCO₃ containing 1 mM MgCl₂ pH 9.8). Color development was stopped by immersing the filter in H₂O₂.

Immunohistochemistry

Serial sections of 5 μm were prepared, mounted on Histostik (Accurate Chemical & Scientific Corp., Westbury, NY, USA) coated slides, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 30% H₂O₂ and absolute methanol (1:9 v/v). Tissue sections were treated first with goat serum and then incubated with the corresponding antibodies followed by biotinylated goat anti-rabbit antibody, enzyme conjugated and chromogen mixture (Zymed, San Francisco, CA, USA) following the directions of the manufacturers.

Immunocytochemistry

Two days postnatal mouse molars were fixed for 4 h in Karnovsky’s fixative as described previously (Dekiewsch et al., 1993). Subsequently, molar tooth organs were decalcified for one week in 4.13% disodium EDTA, dehydrated in graded ethanol, infiltrated and embedded into Epon 812. A second group of mouse molars was treated identically but not decalcified. Thin sections (80 μm) were cut and processed for postembedding immunocytochemistry. Grid-mounted tissue sections were floated in serum blocking solution, washed with PBS and then incubated for 1 h at room temperature on a drop of diluted rabbit anti-tuftelin IgG at a concentration of 1:500. Sections were rinsed again with PBS and then incubated for 20 min with gold-conjugated anti-rabbit IgG. Subsequently, sections were
washed thoroughly with PBS, rinsed with distilled water and contrasted for 5 min with 1% uranylacetate. Sections were rinsed with distilled water and air dried.

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