Int. J. Dev. Biol. 41: 27-38 (1997)

Original Article

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

MARGARITA ZEICHNER-DAVID[1]*, HANG VO[1], HENRY TAN[1], THOMAS DIEKWISCH[2], BRETT BERMAN[1], FLAVIA THIEMANN[1], M[a] DOLORES ALCOCER[1], PATTY HSU[1], TRACY WANG[1], JULIA EYNA[1], JAVIER CATON[1], HAROLD C. SLAVKIN[3] and MARY MacDOUGALL[4]

[1]*Center for Craniofacial Molecular Biology, University of Southern California School of Dentistry, Los Angeles, CA,* [2]*University of Texas at Dallas, School of Dentistry, Dallas, TX,* [3]*Craniofacial Development Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD and* [4]*University of Texas Health Science Center at San Antonio, School of Dentistry, San Antonio, TX, USA*

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 (enamelins, tuft proteins, 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, 1985, 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 (enamelins, 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 amelob-

***Address for reprints:** Center for Craniofacial Molecular Biology, USC School of Dentistry, 2250 Alcazar Street, CSA 106, Los Angeles, CA 90033, USA. FAX: 213.342-2981. e-mail: zeichner@zygote.hsc.usc.edu

0214-6282/97/$05.00

Printed in Spain

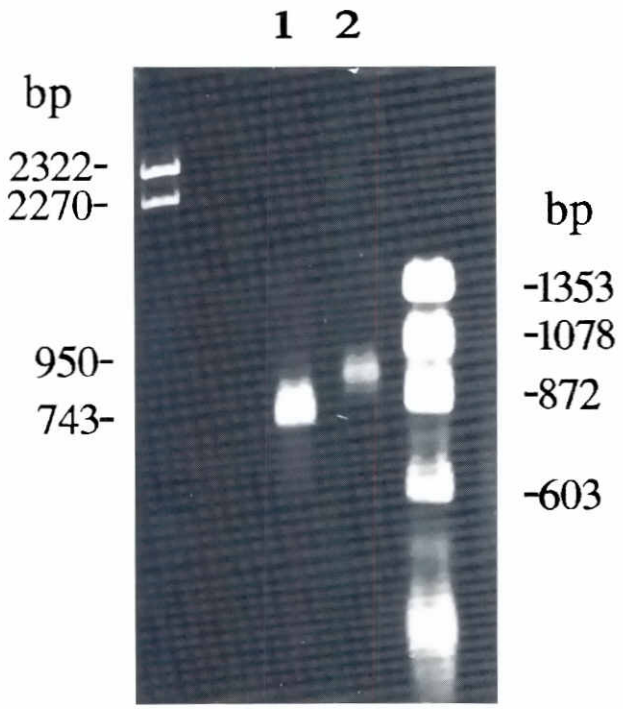

Fig. 1. PCR amplification of mouse tuftelin mRNA. *One μg of poly (A) RNA obtained from newborn mice mandibular first molars was used for RT-PCR using different sets of tuftelin primers. An aliquot of 20 μl of the reaction mixture was subjected to agarose gel electrophoresis followed by staining with ethidium bromide.* **(1)** *Primers 257s and 1000as;* **(2)** *38s and 1000as, resulted in the amplification of a 743bp and a 950bp fragments, respectively.*

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 Lyngstadaas *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/amelin, 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

```
          10          20          30          40          50          60          70          80
     *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CGT AAC TGG TGT ACC TTG GTG GAC GTG CAC CCG GAG AGC CAG ACC GCG GGC AGC GTG GAC ATT CTC AGG CTG ACG CTC CAG
GCA TTG ACC ACA TGG AAC CAC CTG CAC GTG GGC CTC TCG GTC TGG CGC CCG TCG CAC CTG TAA GAG TCC GAC TGC GAG GTC
Arg Asn Trp Cys Thr Leu Val Asp Val His Pro Glu Ser Gln Thr Ala Gly Ser Val Asp Ile Leu Arg Leu Thr Leu Gln

          90          100         110         120         130         140         150         160
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AGT GAA CTG ACA GGA GAT GAA CTT GAG CAC ATA GCC CAG AAG GCG GGC AGG AAG ACC TAT GCC ATG ATG CCA GGC CAC TCC
TCA CTT GAC TGT CCT CTA CTT GAA CTC GTG TAT CGG GTC TTC CGC CCG TCC TTC TGG ATA CGG TAC TAC GGT CCG GTG AGG
Ser Glu Leu Thr Gly Asp Glu Leu Glu His Ile Ala Gln Lys Ala Gly Arg Lys Thr Tyr Ala Met Met Pro Gly His Ser

170         180         190         200         210         220         230         240         25
*     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TCA GGT CAT TCT CTG GCT TCA GAA CTG GTG GAG TCC CAT GAT GGC CAT GAA GAG ATC ATT AAG GTT TAC TTG AAA GGG AGG
AGT CCA GTA AGA GAC CGA AGT CTT GAC CAC CTC AGG GTA CTA CCG GTA CTT CTC TAG TAA TTC CAA ATG AAC TTT CCC TCC
Ser Gly His Ser Leu Ala Ser Glu Leu Val Glu Ser His Asp Gly His Glu Glu Ile Ile Lys Val Tyr Leu Lys Gly Arg

260         270         280         290         300         310         320         330         340
*     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TCT GGG GAC AAG ATG ACC CAT GAG AAG AAC ATC GAC CAG CTG AAG AGT GAG GTC CAG TAC ATC CAG GAG GCC AGG AAC TGC
AGA CCC CTG TTC TAC TGG GTA CTC TTC TTG TAG CTG GTC GAC TTC TCA CTC CAG GTC ATG TAG GTC CTC CGG TCC TTG ACG
Ser Gly Asp Lys Met Thr His Glu Lys Asn Ile Asp Gln Leu Lys Ser Glu Val Gln Tyr Ile Gln Glu Ala Arg Asn Cys

350         ,360        370         380         390         400         410         420         43
*     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CTG CAG AAG CTC CGG GAG GAT ATC AGT AGC AAG CTT GAC AGG AGT CCA GGA GAC CCT CTG CGT CAG CAG GAG ATA CAG GTG
GAC GTC TTC GAG GCC CTC CTA TAG TCA TCG TTC GAA CTG TCC TCA GGT CCT CTG GGA GAC GCA GTC GTC CTC TAT GTC CAC
Leu Gln Lys Leu Arg Glu Asp Ile Ser Ser Lys Leu Asp Arg Ser Pro Gly Asp Pro Leu Arg Gln Gln Glu Ile Gln Val

          440         450         460         470         480         490         500         510
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTA CTA GAA AAG CCA AAT GGC TTT AGT CAG AGT CCC ATG ACT CTC TAC AGT AGC CCA CCT GAG GTG GAC CCT AGC ATG AGT
CAT GAT CTT TTC GGT TTA CCG AAA TCA GTC TCA GGG TAC TGA GAG ATG TCA TCG GGT GGA CTC CAC CTG GGA TCG TAC TCA
Val Leu Glu Lys Pro Asn Gly Phe Ser Gln Ser Pro Met Thr Leu Tyr Ser Ser Pro Pro Glu Val Asp Pro Ser Met Ser

          520         530         540         550         560         570         580         590
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAA GAT GTT GAG AGC TTG AAG AAG ACT GTG CAG GAG TTG CTT GTC AAG CTG CGG GAG GCT GAG CGG CGA CAC CAG TCA GAC
CTT CTA CAA CTC TCG AAC TTC TTC TGA CAC GTC CTC AAC GAA CAG TTC GAC GCC CTC CGA CTC GCC GCT GTG GTC AGT CTG
Glu Asp Val Glu Ser Leu Lys Lys Thr Val Gln Glu Leu Leu Val Lys Leu Arg Glu Ala Glu Arg Arg His Gln Ser Asp

          600         610         620         630         640         650         660         670
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CGT GTG GCC TTT GAG GTC ACA CTC AGC CGT TAT CAG CGA GAA GCA GAA CAG AGT AAT GTG GCC CTT CAG AGA GAG GAG GAC
GCA CAC CGG AAA CTC CAG TGT GAG TCG GCA ATA GTC GCT CTT CGT CTT GTC TCA TTA CAC CGG GAA GTC TCT CTC CTC CTG
Arg Val Ala Phe Glu Val Thr Leu Ser Arg Tyr Gln Arg Glu Ala Glu Gln Ser Asn Val Ala Leu Gln Arg Glu Glu Asp

          680         690         700         710         720         730         740         750
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AGA GTG GAG CAG AAA GCG GCA GAA ATC GAG GAG CTA CAG AGG CGC TTG CTG GGG ATG GAG GCG GAG CAT CAG GCC TTA CTG
TCT CAC CTC GTC TTT CGC CGT CTT TAG CTC CTC GAT GTC TCC GCG AAC GAC CCC TAC CTC CGC CTC GTA GTC CGG AAT GAC
Arg Val Glu Gln Lys Ala Ala Glu Ile Glu Glu Leu Gln Arg Arg Leu Leu Gly Met Glu Ala Glu His Gln Ala Leu Leu

          760         770         780         790         800         810         820         830
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTG AAA GTC AGG GAA GGA GAG ATG GCC CTA GAA GAG CTT CGG ATC AAG AAC GCT GAC TGT CAA ACA GAA AGA GAA AAG TCT
CAC TTT CAG TCC CTT CCT CTC TAC CGG GAT CTT CTC GAA GCC TAG TTC TTG CGA CTG ACA GTT TGT CTT TCT CTT TTC AGA
Val Lys Val Arg Glu Gly Glu Met Ala Leu Glu Glu Leu Arg Ile Lys Asn Ala Asp Cys Gln Thr Glu Arg Glu Lys Ser

          840         850         860         870         880         890         900         910
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GCT GCT CTA GAA AAG GAA GTG GCT GGG TTT CGG GAG AAG ATC CAC CAC TTG GAT GAC ATG CTG AAG AGC CAG CAG CGG AAA
CGA CGA GAT CTT TTC CTT CAC CGA CCC AAA GCC CTC TTC TAG GTG GTG AAC CTA CTG TAC GAC TTC TCG GTC GTC GCC TTT
Ala Ala Leu Glu Lys Glu Val Ala Gly Phe Arg Glu Lys Ile His His Leu Asp Asp Met Leu Lys Ser Gln Gln Arg Lys

          920         930         940         950         960         970         980
    *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTT CGG CAG ATG ATA GAG CAG CTC CAG AAT TCA AAA GCT GTG ATC CAG TCA AAG GAT GCC ACC ATT CAA GA
CAA GCC GTC TAC TAT CTC GTC GAG GTC TTA AGT TTT CGA CAC TAG GTC AGT TTC CTA CGG TGG TAA GTT CT
Val Arg Gln Met Ile Glu Gln Leu Gln Asn Ser Lys Ala Val Ile Gln Ser Lys Asp Ala Thr Ile Gln Xxx
```

Fig. 2. DNA and deduced protein sequence of mouse tuftelin clone mT57

(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.

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 QSKDTTIQELKEKIA was produced in rabbits. The specificity of the antibody was first characterized by western blot immunostaining using 5 day-old mouse tooth enamel proteins

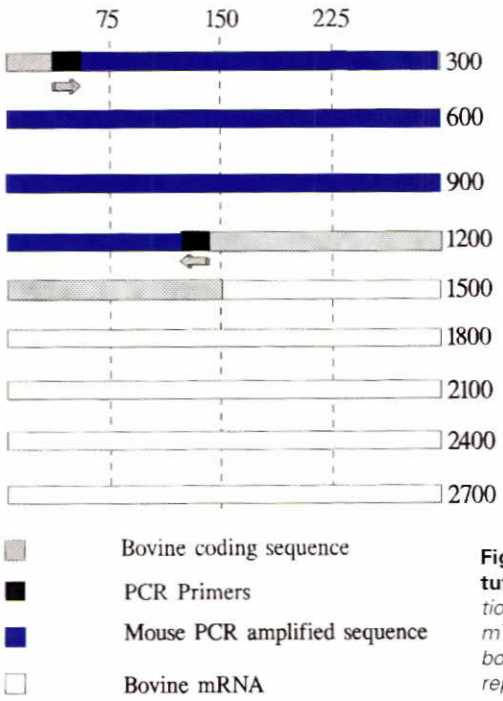

Fig. 3. Map of bovine tuftelin. *Representation of mouse clone mT57 in relation to the bovine tuftelin cDNA reported by Deutsch et al. (1991).*

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 is tuftelin 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 immunonegative 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).

```
M     1 RNWCTLVDVH PESQTAGSVD ILRLTLQSEL TGDELEHIAQ KAGRKTYAMV SSHSTSHSLA
B    13 RNWCTLVDVH PEGQTAGSVD VLRLTLQSEL TGDELEHIAQ KAGRKTYAMM PGHSSGHSLA

B    61 SELVESNDGH EEIIKVYLKG RSGDKMIHEK NINQLKSEVQ YIQEARNCLQ KLREDISSKL
M    73 SELVESHDGH EEIIKVYLKG RSGDKMTHEK NIDQLKSEVQ YIQEARNCLQ KLREDISSKL

M   121 DRSPGDPLRQ QEIQVVLEKP NGFSQSPMTL YSSPPEVDPS MSEDVESLKK TVQELLVKLR
B   133 DRDPGDSVHK QEIQVVLEKQ NGLSEGPLTT YSSPPEVDTH INEDVESLRK TVQDLLVKLQ

M   181 EAERRHQSDR VAFEVTLSRY QREAEQSNVA LQREEDRVEQ KAAEIEELQR RLLGMEAEHQ
B   193 EAEQQHQSDC SAFKVTLSQY QREAKQSQVA LQRAEDRAEQ KEAEVGELQR RLQGMETDYQ

M   241 ALLVKVREGE MALEELRIKN ADCQTEREKS AALEKEVAGF REKIHHLDDM LKSQQRKVRQ
B   253 AILAKVREGE TALEELRSKN VDCQAEQEKA ANLEKEVAGL REKIHHLDDM LKSQQRKVRQ

M   301 MIEQLQNSKA VIQSKDATIQ
B   313 MIEQLQNSKA VIQSKDTTIQ
```

Fig. 4. Homology of mouse and bovine tuftelin. *Deduced amino acid sequences of bovine tuftelin (B) and mouse tuftelin (M).*

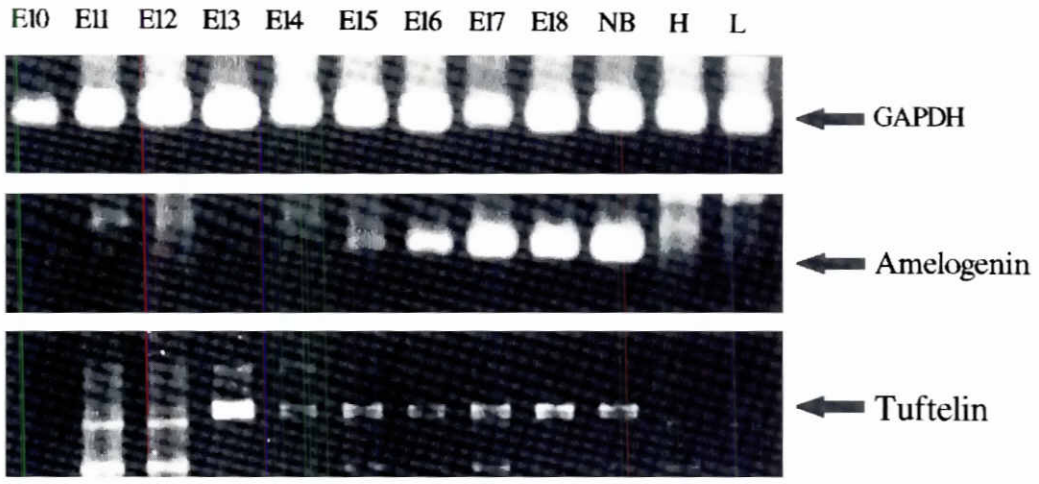

Fig. 5. Expression of tuftelin and amelogenin transcripts at different developmental stages. *Poly (A) RNA extracted from mouse mandibles obtained from 10 days gestation (E10) through NB mice was subjected to RT-PCR using GAPDH, amelogenin and tuftelin primers. H, heart mRNA; L, liver mRNA.*

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

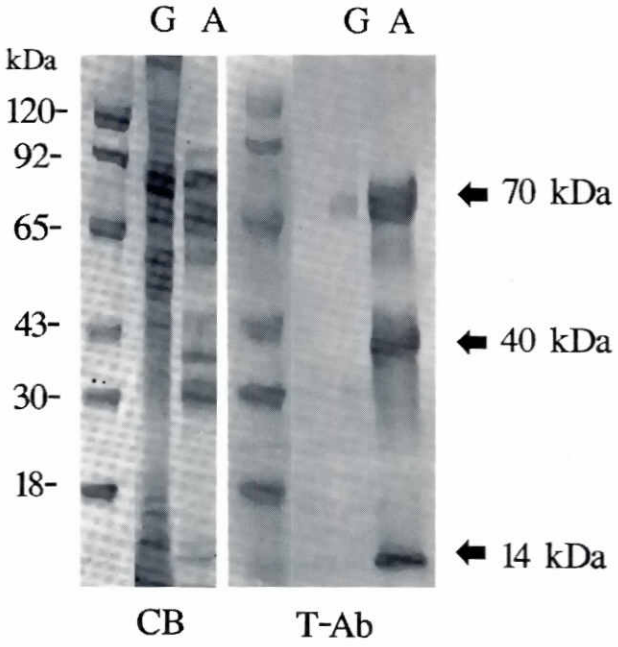

Fig. 6. Western blot immunodetection of tuftelin. *Mouse enamel proteins extracted with 0.5 M acetic acid (A) or 4 M GuHCl-0.5M EDTA (G) were fractionated in 12% polyacrylamide SDS gels in duplicate gels. One of the gels was stained with Coomassie blue R250 and the other gel transferred to nitrocellulose for western blot immunostaining with the tuftelin antibody (T-Ab).*

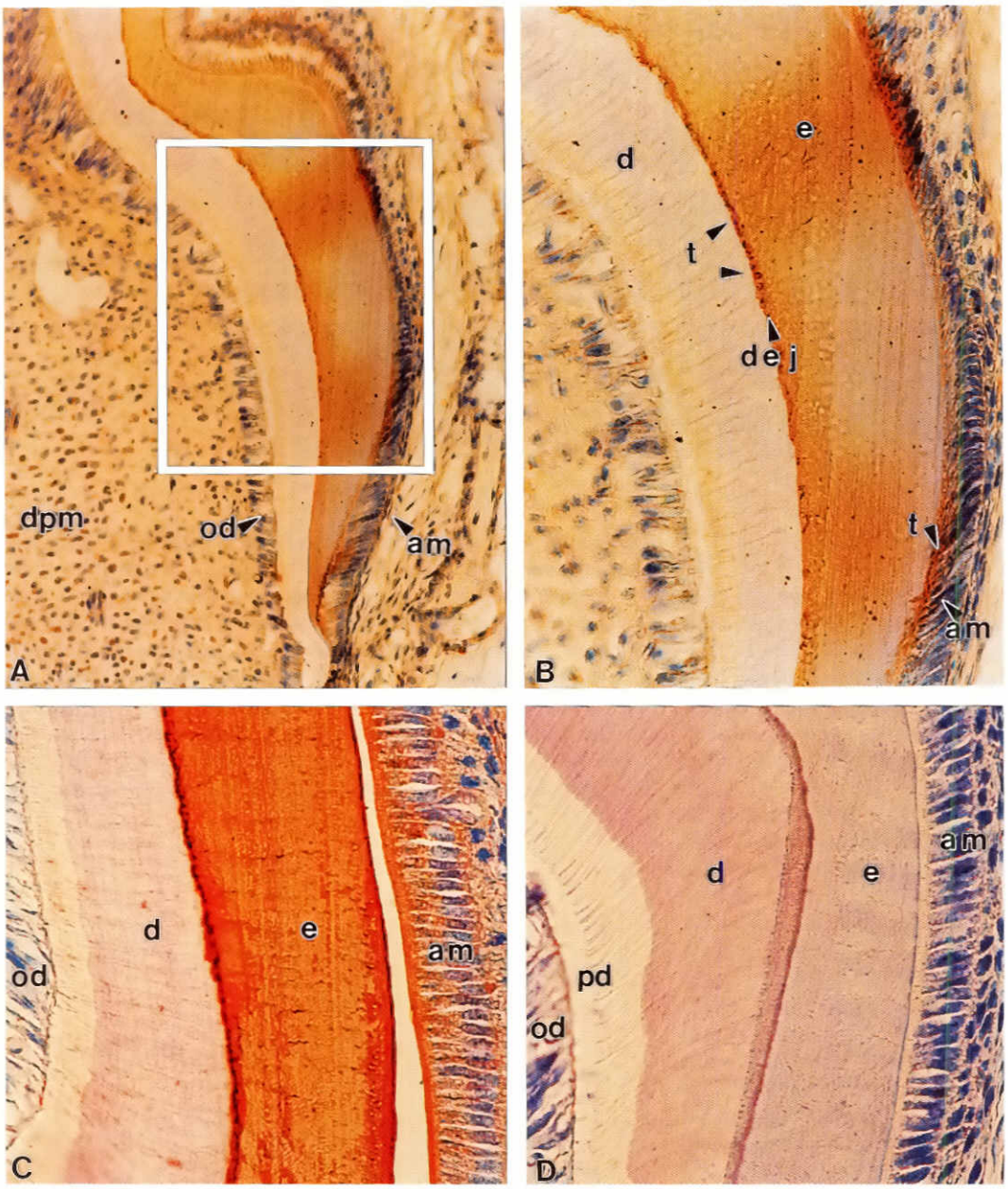

Fig. 7. Immunolocalization of tuftelin in mouse molars. *Paraffin sections prepared from 7-day-old mouse mandibles were incubated with an IgG fraction (3 mg/ml) of the tuftelin anti-peptide antibody at a 1:40 dilution* **(A and B)**. *Strong immunolocalization of tuftelin antigens was seen in the dentino-enamel junction (DEJ) as well as in the enamel extracellular matrix (e).* **(C)** *Similar tissue stained with an amelogenin antibody (1:1000 dilution).* **(D)** *A control (no primary antibody). Abbreviations: dpm, dental papillae mesenchyme; od, odontoblasts; am, ameloblasts; d, dentin; t, tuftelin; dej, dentino-enamel junction; e, enamel; pd, pre-dentin.*

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 enamelins and were similar to studies reported by Herold *et al.* (1990) and Hayashi *et al.* (1986) using monoclonal antibodies against non defined enamelins.

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).

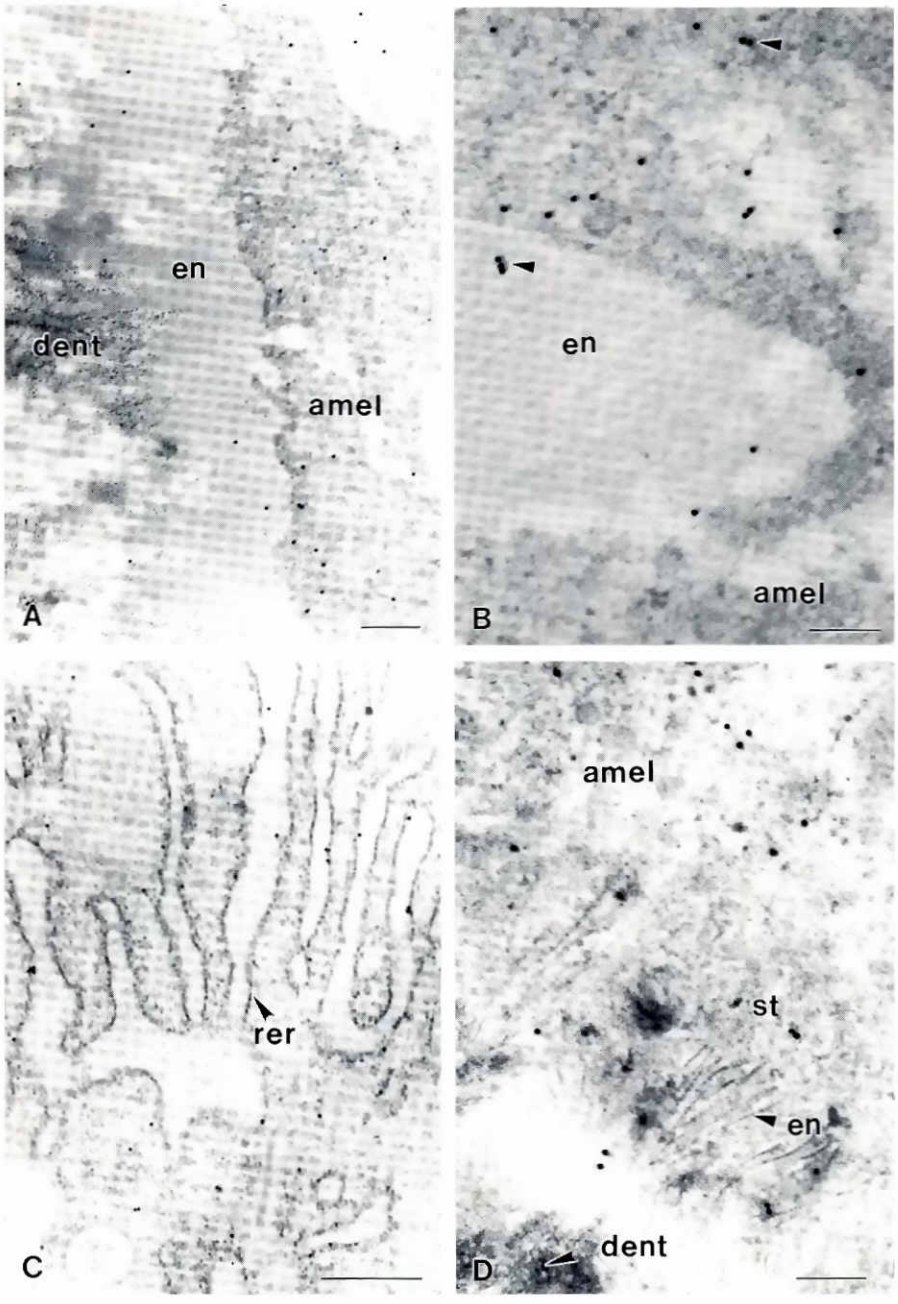

Fig. 8. Protein A-gold high resolution immunocytochemistry. *Two-day postnatal mouse molars were sectioned and processed for postembedding immunocytochemistry.* **(A)** *Represents the forming dentino-enamel junction.* **(B)** *Section through the Tomes' processes.* **(C)** *Represents the ameloblast rough endoplasmic reticulum and* **(D)** *represents the dentino-enamel junction area of a decalcified tissue. Abbreviations: dent, dentin; en, enamel; amel, ameloblast; rer, rough endoplasmic reticulum.*

Couwenhoven and Snead (1994) recently showed that if the enamel organ epithelia 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,

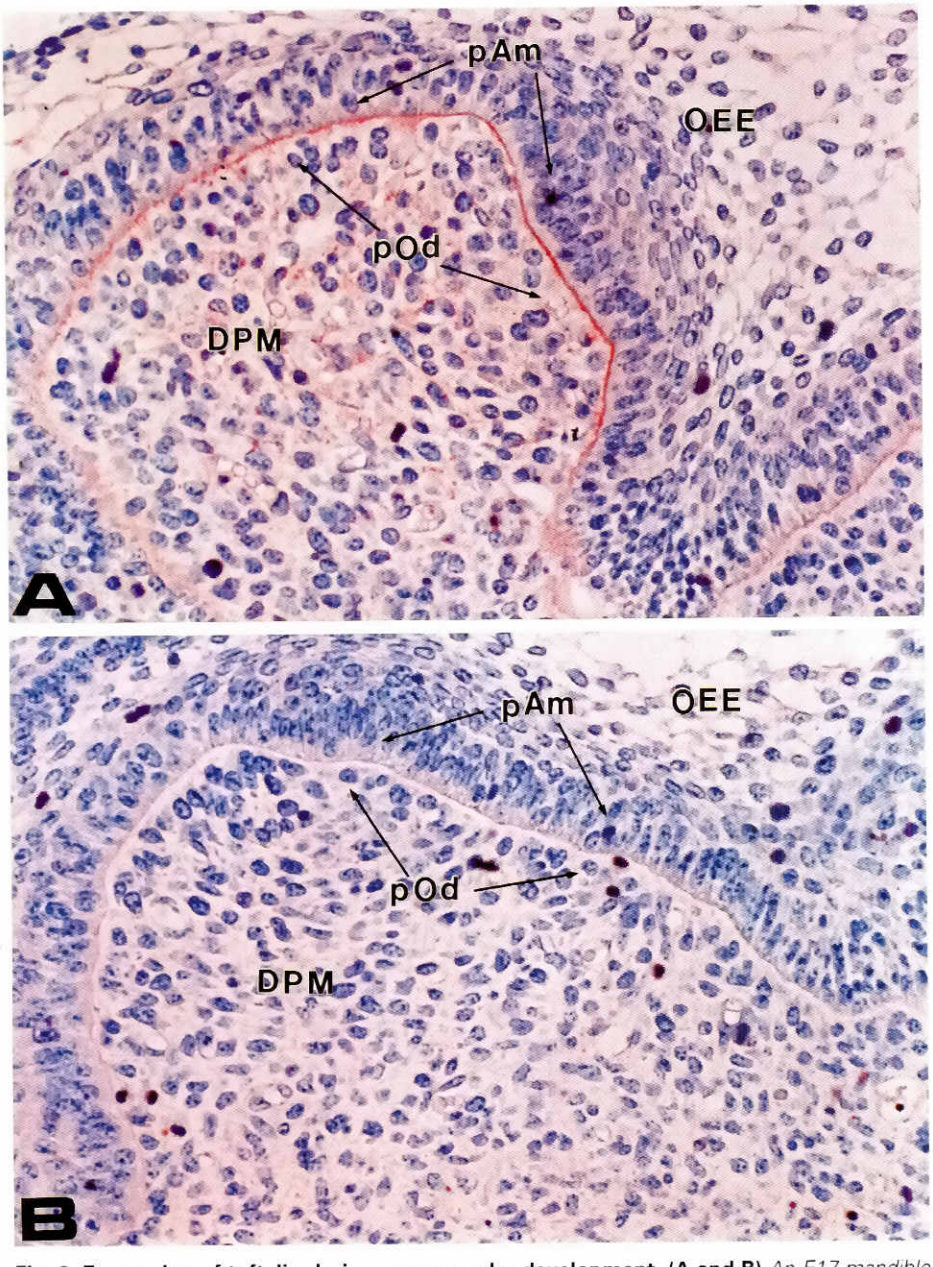

Fig. 9. Expression of tuftelin during mouse molar development. (A and B) *An E17 mandible incubated with a 1:40 dilution of the tuftelin antibody* **(A)** *or no primary antibody* **(B)**. *DPM, dental papillae mesenchyme; ECM, extracellular matrix; OEE, outer enamel epithelia; pAM, pre-ameloblast cells;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

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 mRNA extraction were dissected, immediately frozen in dry ice and kept at -90°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 Microfastrack 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 d(T)-cellulose. RNA concentration is determined by A260nm (1 O.D. 260= 40 μg RNA)

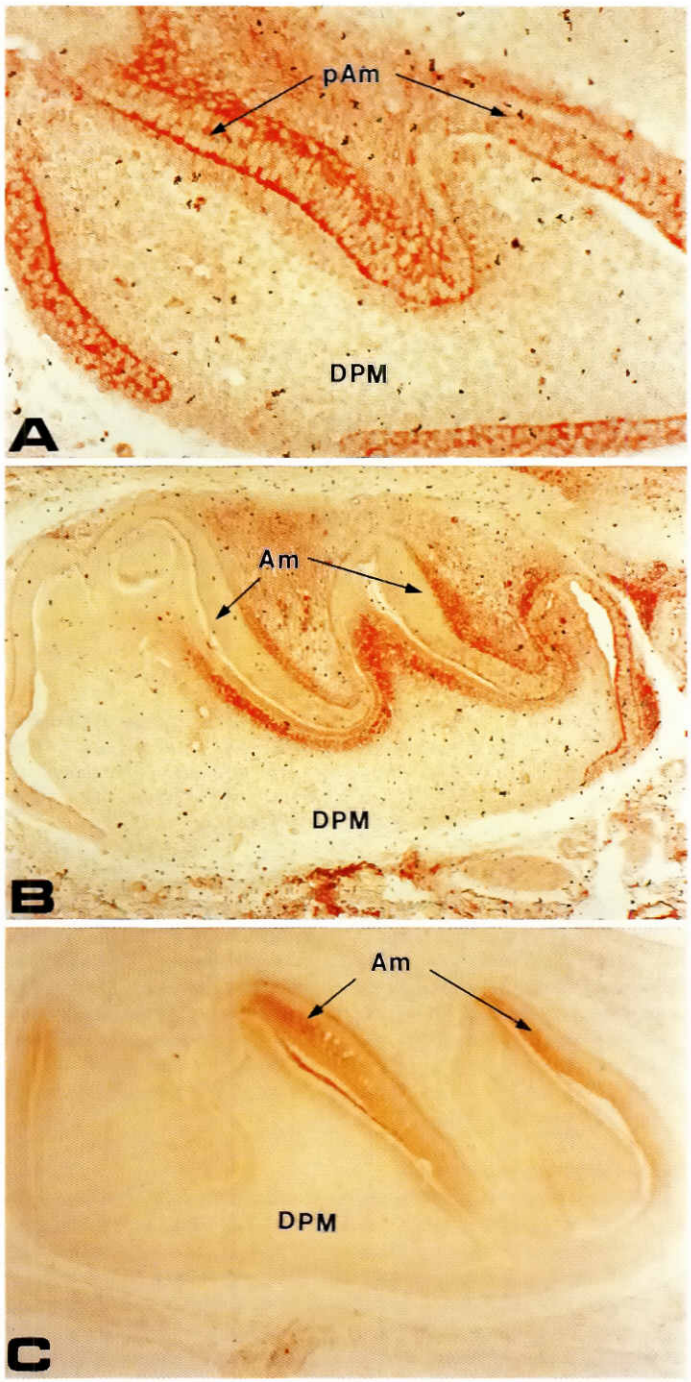

Fig. 10. Expression of cyclin A during tooth development. *Mouse mandibular molars incubated with anti-cyclin A antibody at E17* **(A)** *and newborn* **(B)** *stages of development.* **(C)** *Represents newborn molar incubated with an amelogenin antibody. DPM, dental papillae mesenchyme; Am, ameloblast 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 manufacturers. 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 [38s= CGTAACTGGTGTACCTTG, 259s= TAAGGTGTACTTGAAGGGG and 1000as= GTCGGCAATCTTCTCCTTGAGC (Deutsch *et al.*, 1991)], mouse amelogenin (5'= AACCAATGATGCCCGTTCC and 3'= CTTTTAATCCACTTCCTCCCGC) or rat glycerol phosphate dehydrogenase [(GAPDH) 5'= TGACATCAAGAAGGTGAAG and 3'= TCCTTGGAGGCCATGTAGGCC] 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 pCR™II vector for TA-cloning (Invitrogen, San Diego, CA, USA) to obtain the full sequence. Epicurean Coli Sure™ supercompetent 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

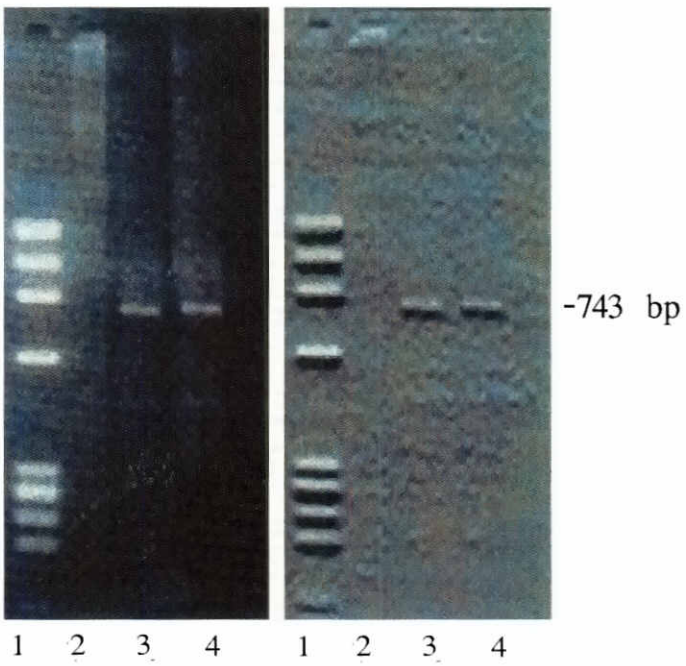

Fig. 11. Expression of tuftelin by dental papillae mesenchyme cells. *Tuftelin primers were used to amplify liver mRNA (2), whole mouse molar mRNA obtained from newborn mouse (3) or DPM cells mRNA (4). All RT-PCR reactions were initiated with 1 µg of poly (A)-RNA extracted from the respective tissues.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

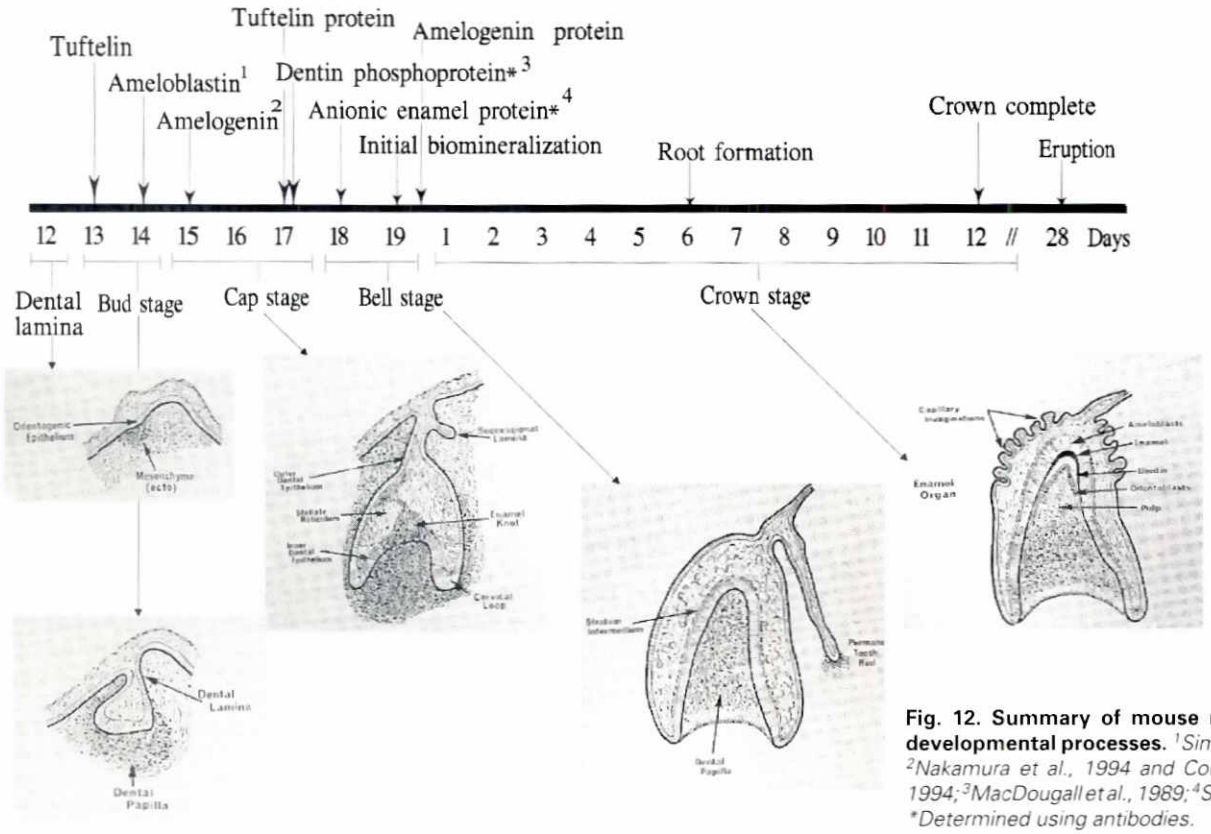

Fig. 12. Summary of mouse molar sequential developmental processes. *[1]Simmons et al., 1996; [2]Nakamura et al., 1994 and Couwenhoven et al., 1994; [3]MacDougall et al., 1989; [4]Slavkin et al., 1988a. *Determined using antibodies.*

colony from the plate and placed in 200 µl water and boiled for 10 min and then cooled in ice for 5 min. The procedure is repeated one more time. An aliquot of 10 µl of the lysate is taken for PCR amplification using flanking primers (M13 reverse and forward). The products are analyzed by agarose gel electrophoresis and stained with ethidium bromide. The clones containing inserts are grown in liquid cultures (10 ml LB containing Ampicillin) and the plasmid DNA is obtained in mini preparations using the method of Birnboim and Doly (1979). DNA sequence is determined following the Dideoxy method described by Sanger *et al.* (1977). The reaction is performed using Sequenase version 2.0 (USB) in the presence of [^{35}S]-dATP following the protocol described by the manufacturers. IBI's MacVector software was used to search the Entrez nucleic acid data base for sequence homology.

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 QSKDTTIQELKEKIA (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 $NaHCO_3$ containing 1 mM $MgCl_2$ pH 9.8). Color development was stopped by immersing the filter in H_20.

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% H_2O_2 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 (Diekwisch *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.

Acknowledgments

The authors thank Dr. Fred Hall for providing the antibody against cyclin A. We also want to thank Mr. Pablo Bringas Jr. and Mr. Valentino Santos for all their assistance in this work. This work was supported by research grant DE-02848 and DE11172, NIDR, NIH, USPHS.

References

AHMAD, N. and RUCH, J.V. (1987). Comparison of growth and cell proliferation kinetics during mouse molar odontogenesis *in vivo* and *in vitro*. *Cell Tissue Kinet. 20:* 319-329.

AMAR, S., LUO, W., SNEAD, M.L. and RUCH, J.V. (1989). Amelogenin gene expression in mouse incisor heterotypic recombinations. *Differentiation 41:* 56-61.

AOBA, T., FUKAE, M., TANABE, T., SHIMIZU, M. and MORENO, E.C. (1987). Selective adsorption of porcine-amelogenins onto hydroxyapatite and their inhibitory activity on hydroxyapatite growth in supersaturated solutions. *Calcif. Tissue Int. 41:* 281-289.

AOBA, T., MORENO, E.C., KRESAK, M. and TANABE, T. (1989). Possible roles of partial sequences at N- and C-terminal of amelogenin in protein-enamel mineral interaction. *J. Dent. Res. 68:* 1331-1336.

BIRNBOIM, H.C. and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res. 7:* 1513-1523.

CARBONARO-HALL, D., WILLIAMS, R., WU, L., WARBURTON, D., ZEICHNER-DAVID, M., MACDOUGALL, M., TOLO, V. and HALL, F. (1993). G1 expression and multistage dynamics of cyclin A in human osteosarcoma cells. *Oncogene 8:* 1649-1659.

CERNY, R., SLABY, I., HAMMARSTROM, L. and WURTZ, T. (1996). A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J. Bone Miner. Res. 11:* 883-891.

COHN, S.A. (1957). Development of the molar teeth in the Albino mouse. *Am. J. Anat. 101:* 295-319.

COUWENHOVEN, R. and SNEAD, M.L. (1994). Early determination and permissive expression of amelogenin transcription during mouse mandibular first molar development. *Dev. Biol. 164:* 290-299.

DEUTSCH, D. (1989). Structure and function of enamel proteins. *Anat. Rec. 224:* 189-210

DEUTSCH, D., PALMON, A., FISHER, L.W., KOLODNY, A., TERMINE, J.D. and YOUNG, M.F. (1991). Sequencing of bovine enamelin ("Tuftelin") a novel acidic enamel protein. *J. Biol. Chem. 266:* 16021-16028.

DEUTSCH, D., SHAPIRA, L., AYALOFF, A., LEVIEL, D., YOELI, Z. and ARAD, A. (1984). Protein and mineral changes during prenatal and postnatal development and mineralization of human deciduous enamel. In *Tooth Enamel IV* (Eds. R.W. Fearnhead and S. Suga). Elsevier/North Holland, Amsterdam, pp. 234-239.

DIEKWISCH, T., DAVID, S., BRINGAS, P., SANTOS, V. and SLAVKIN, H.C. (1993). Antisense inhibition of AMEL translation demonstrates supramolecular controls for enamel HAP crystal growth during embryonic mouse molar development. *Development 117:* 471-482.

DOI, Y., EANES, E.D., SHIMOKAWA, H. and TERMINE, J.D. (1984). Inhibition of seeded growth of enamel apatite crystals by amelogenin and enamelin proteins *in vitro*. *J. Dent. Res. 63:* 98-105.

FEARNHED R.W. (1979). Matrix-mineral relationships in enamel tissues. *J. Dent. Res. 58B:* 909-916.

FINCHAM, A., LAU, E.C., SIMMER, J. and ZEICHNER-DAVID, M. (1992). Amelogenin biochemistry form and function. In *Proceedings of the Fourth International Conference on Chemistry and Biology of Mineralized Tissues* (Eds. H.C. Slavkin and P. Price). Elsevier, Amsterdam, pp. 187-201.

FINCHAM, A.G. and BELCOURT, A.B. (1985). Amelogenin biochemistry: current concepts. In *The Chemistry and Biology of Mineralized Tissues* (Ed. W.T. Butler). Ebsco Media Inc., Birmingham, p. 241.

FINCHAM, A.G., BELCOURT, A.B. and TERMINE, J.D. (1982). Changing patterns of enamel matrix proteins in the developing bovine tooth. *Caries Res. 16:* 64-71.

GEORGE, A., SABSAY, B., SIMONIAN, P. and VEIS, A. (1993). Characterization of a novel dentin matrix acidic phosphoprotein: implications for induction of biomineralization. *J. Biol. Chem. 268:* 12624-12630.

GLIMCHER, M.J. (1979). Phosphopeptides of enamel. *J. Dent. Res. 58B:* 790-806.

HAYASHI, Y., BIANCO, P., SHIMOKAWA, H., TERMINE, J.D. and BONUCCI, E. (1986). Organic-inorganic relationships, and immunohistochemical localization of amelogenins and enamelins in developing enamel. *Basic Appl. Histochem. 30:* 291-299.

HEROLD, R., ROSENBLOOM, J. and GRANOVSKY, M. (1989). Phylogenetic distribution of enamel proteins: immunohistochemical localization with monoclonal antibodies indicates the evolutionary appearance of enamelins prior to amelogenins. *Calcif. Tissue Int. 45:* 88-94.

KALLENBACH, E. (1971). Electron microscopy of the differentiating rat incisor ameloblast. *J. Ultrastruct. Res. 35:* 508-531.

KALLENBACH, E. (1974). Fine structure of rat incisor ameloblast in transition between enamel secretion and maturation stages. *Tissue Cell 6:* 173

KOLLAR, E.J. (1983). Epithelio-mesenchymal interactions in the mammalian integument: tooth development as a model for instructive interaction. In *Epithelial-Mesenchymal Interactions in Development* (Eds. R.H. Sawyer and J.F. Fallon). Praeger Publishers, New York, pp. 87-102.

KREBSBACH, P.H., LEE, S.K., MATSUKI, Y., KOZAK, C.A., YAMADA, R.M. and YAMADA, Y. (1996). Full-length sequence, localization, and chromosomal mapping of ameloblastin – a novel tooth-specific gene. *J. Biol. Chem. 271:* 4431-4435.

LIMEBACK, H. and SIMIC, A. (1989). Porcine high molecular weight enamel proteins are primarily stable amelogenin aggregates and serum albumin-derived proteins. In *Tooth Enamel V* (Ed. R. Fearnhead). Florence Publishers, Yokohama, pp. 269-277.

LOWENSTAM, H.A. and WEINER, S. (1989). *On Biomineralization.* Oxford University Press, New York-Oxford.

LUMSDEN A.G. (1987). The neural crest contribution to tooth development in the mammalian embryo. In *Developmental and Evolutionary Aspects of the Neural Crest* (Ed. P.F.A. Maderson). John Wiley & Sons, New York, pp. 261-300.

LUMSDEN, A.G.S. (1988), Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth. *Development 103 (Suppl.):* 55-169.

LYNGSTADAAS, S.P., RISNES, S., SPROAT, B.S., THRANE, P.S. and PRYDZ, H.P. (1996). A synthetic, chemically-modified ribozyme eliminates amelogenin, the major translation product in developing mouse enamel *in vivo*. *EMBO J. 14:* 5224-5229.

MACDOUGALL, M., ZEICHNER-DAVID, M. and SLAVKIN, H. (1989). Characterization of extracellular and nascent dentin phosphoproteins. *Connect. Tissue Res. 22:* 71-77.

MENANTEAU, J., GREGOIRE, M., DALCUSI, G. and JANS, I. (1987). *In vitro* albumin binding on apatite crystals from developing enamel. *J. Dent. Res. 62:* 100-104.

NAKAMURA, M., BRINGAS, P., NANCI, A., ZEICHNER-DAVID, M., ASHDOWN, B.V SLAVKIN, H.C. (1994). Translocation of enamel proteins from inner enamel epithelia to odontoblasts during mouse tooth development. *Anat. Rec. 238:* 383-396.

OKAMURA K. (1983). Localization of serum albumin in dentin and enamel. *J. Dent. Res. 62:* 100-104.

RITCHIE, H.H., HOU, H., VEIS, A. and BUTLER, W.T. (1994). Cloning and sequence determination of rat dentin sialoprotein (DSP), a novel dentin protein. *J. Biol. Chem. 269:* 3698-3702.

RITCHIE, H.H., SHIGEYAMA, Y., STRAYHORN, C., HANKS, C.T., SOMERMAN, M.J. and BUTLER, W.T. (1996). Specific expression of DSP messenger-RNA by odontoblasts. *J. Dent. Res. 75:* 1088-1088.

ROBINSON, C., KIRKHAM, J. and SHORE, R.C. (1989). Extracellular processing of enamel matrix and origin and function of tuft protein. In *Tooth Enamel V* (Ed. R.W. Fearnhead). Florence Publishers, Yokohama, pp. 59-68.

ROBINSON C., LOWE N.R. and WEATHERELL J.A. (1977). Changes in amino-acid composition of developing rat incisor. *Calcif. Tissue Res. 23:* 19-31.

RUCH J.V. (1985). Epithelial-mesenchymal interactions. In *The Chemistry and Biology of Mineralized Tissues* (Ed. W.T. Butler). EBSCO Media, Birmingham (AL), pp. 54-61.

RUCH J.V. (1988). Determinism of odontogenesis. *Cell Biol. Rev. 14:* 1-112.

SANGER, F., NICKLEN, S. and COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:* 5263.

SIMMER, J.P., LAU, E.C., HU, C.C., AOBA, T., LACEY, M., NELSON, D., ZEICHNER-DAVID, M., SNEAD, M.L., SLAVKIN, H.C. and FINCHAM, A.G. (1994). Isolation and characterization of a mouse amelogenin expressed in *Escherichia coli. Calcif. Tissue Int. 54:* 312-319.

SIMMONS, D., GU, T.T., LEACH, R., KREBSBACH, P.H. and MACDOUGALL, M. (1996). Identification and temporal expression of cDNA for murine ameloblastin. *J. Dent. Res. 75:* 73-73

SLAVKIN H.C. (1990). Regulatory issues during early craniofacial development: a summary. *Cleft Palate J. 27:* 101-109.

SLAVKIN, H.C., BESSEM, C., BRINGAS, P., ZEICHNER-DAVID, M., NANCI, A. and SNEAD, M.L. (1988a). Sequential expression and differential function of multiple enamel proteins during fetal, neonatal and early postnatal stages of mouse molar organogenesis. *Differentiation 37:* 26-39.

SLAVKIN, H.C., MACDOUGALL, M., ZEICHNER-DAVID, M., OLIVER, P., NAKAMURA, M. and SNEAD, M.L. (1988b). Molecular determinants of cranial neural crest-derived odontogenic ectomesenchyme during dentinogenesis. *Am. J . Med. Genet. 4:* 7-22.

SLAVKIN, H.C., MINO, W. and BRINGAS, P. (1976). The biosynthesis and secretion of precursor enamel protein by ameloblast as visualized by autoradiography after tryptophan administration. *Anat. Rec. 185:* 289-312.

SLAVKIN, H.C., SNEAD, M.L., ZEICHNER-DAVID, M., JASKOLL, T.F. and SMITH, B.T. (1984). Concepts of epithelial-mesenchymal interactions during development: tooth and lung organogenesis. *J. Cell. Biochem. 26:* 117-125.

SMITH, C.E., CHEN, W.Y., ISSID, M. and FAZEL, A. (1995). Enamel matrix protein-turnover during amelogenesis. Basic biochemical properties of short lived sulfated enamel proteins. *Calcif. Tissue Int. 57:* 133-144.

SNEAD, M.L., BRINGAS, P., BESSEM, C. and SLAVKIN, H.C. (1984). *De novo* gene expression detected by amelogenin gene transcript analysis. *Dev. Biol. 104:* 255-258.

SNEAD, M.L., LAU, E., ZEICHNER-DAVID, M., NANCI, T., BENDAYAN, M., BRINGAS, P., BESSEM, C. and SLAVKIN, H.C. (1987). Relationship of the ameloblast biochemical phenotype to morpho- and cytodifferentiation. In *Molecular Approaches to Developmental Biology* (Eds. A. Firtel and E. Davidson). Alan Liss, New York, pp. 641-652.

SNEAD, M.L., LUO, W., LAU, E.C. and SLAVKIN, H.C. (1988). Spatial- and temporal-restricted pattern for amelogenin gene expression during mouse molar tooth organogenesis. *Development 104:* 77-85.

STRAWICH, E. and GLIMCHER, M.J. (1990). Tooth 'enamelin' identified mainly as serum proteins. Major 'enamelin' is albumin. *Eur. J. Biochem. 191:* 47-56.

STRAWICH, E., SEYER, J. and GLIMCHER, M.J. (1993). Immuno-identification of two non-amelogenin proteins of developing bovine enamel isolated by affinity chromatography. Further proof that tooth "enamelins" are mainly serum proteins. *Connect. Tissue Res. 29:* 163-169.

TERMINE, J.D., BELCOURT, A.B., CHRISTNER, P.J., CONN, K. and MYLEN, M.U. (1980). Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. *J. Biol. Chem. 255:* 9760-9765

THESLEFF, I. and HURMERINTA, K. (1981). Tissue interactions in tooth development. *Differentiation 18:* 75-88

THESLEFF, I., PARTANEN, A.M. and VAINIO, S. (1991). Epithelial-mesenchymal interactions in tooth morphogenesis: the role of extracellular matrix, growth factors, and cell surface receptors. *J. Craniofac. Genet. Dev. Biol. 11:* 229-237.

THESLEFF, I., VAAHTOKARI, A. and VAINIO, S. (1990). Molecular changes during determination and differentiation of the dental mesenchymal cell lineage. *J. Biol. Buccale 18:* 179-188.

WINTER, G.B., and BROOK, A.H. (1975). Enamel hypoplasia and anomalies of the enamel. *Dent. Clin. North Am. 19:* 3-24.

WITKOP, C.J. and SAUK, J.J. (1976). Heritable defects of enamel. In *Oral Facial Genetics* (Eds. R.E. Steward and G.H. Prescott). The C.V. Mosby Co., St. Louis, pp. 151-226.

ZEICHNER-DAVID, M., DIEKWISCH, T., FINCHAM, A., LAU, E., MACDOUGALL, M., MORADIAN-OLDAK, J., SIMMER, J., SNEAD, M. and SLAVKIN, H.C. (1995). Control of ameloblast cell differentiation. *Int. J. Dev. Biol. 39:* 69-92.

ZEICHNER-DAVID, M., MACDOUGALL, M., VIDES, J., SNEAD, M.L., SLAVKIN, H.C., TURKEL, S.B. and PAVLOVA, Z. (1987). Immunochemical and biochemical studies of human enamel proteins during neonatal development. *J. Dent. Res. 66:* 50-56.

Accepted for publication: November 1996