Int. J. Dev. Biol. 40: 1141-1150 (1996)

Original Article

Ameloblastin expression in rat incisors and human tooth germs

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ABSTRACT We recently identified ameloblastin as an ameloblast-specific gene product from a rat incisor cDNA library (Krebsbach *et al., J. Biol. Chem. 271:* 4431-4435, 1996). Here we report the developmental pattern of expression of ameloblastin in rat incisors and human tooth germs as visualized by *in situ* hybridization and immunochemistry. Compared to the expression of amelogenin, the major ameloblast product, ameloblastin mRNA was more widely expressed in ameloblasts from the presecretory to the late maturation stage of development. Ameloblastin mRNA was first observed in the juxtanuclear cytoplasm of presecretory stage ameloblasts, gradually increased in the distal cytoplasm of secretory stage ameloblasts and was found throughout the cytoplasm of early to late maturation stage ameloblasts. The immunostaining of ameloblastin, using a monospecific antibody raised against a recombinant protein, showed intense reactivity in Tomes' processes of secretory stage ameloblasts and surrounding enamel. The immunoreaction was concentrated in the juxtanuclear cytoplasm of late maturation stage ameloblasts. High-resolution colloidal gold immunocytochemistry established the presence of ameloblastin antigenicity in the Golgi apparatus, secretory granules in Tomes' process and enamel. Human tooth germs in early to late bell stage also expressed ameloblastin mRNA and ameloblastin antigenicity in the ameloblasts. Western blot analysis of protein extracts from rat incisor tissues indicated that ameloblastin can be found in the enamel epithelial tissue and in mineralized enamel, as well as in the EDTA decalcification solution. These data indicate that ameloblastin is an ameloblast secretory product which is sequentially expressed from the presecretory to the late maturation stage in rat and human teeth. This unique developmental pattern suggests that ameloblastin may have a broader role in amelogenesis than amelogenin and tuftelin.

KEY WORDS: *ameloblastin, amelogenesis, enamel protein, tooth*

Introduction

Tooth organogenesis results from reciprocal interactions between neural crest-derived ectomesenchyme and oral epithelium (Lumsden, 1988; Kollar and Mina, 1991; Ruch *et al.*, 1995; Zeichner-David *et al.*, 1995). Specialized cells called odontoblasts and ameloblasts produce the dentin extracellular matrix (dentinogenesis) and the enamel extracellular matrix (amelogenesis), respectively. Amelogenesis is characterized by the stage- and spatial-specific regulation of different enamel proteins (Slavkin *et al.*, 1988; Snead *et al.*, 1988; Inage *et al.*, 1989; Ruch *et al.*, 1995). The rat incisor is a useful system to study the molecular mechanism underlying amelogenesis since the continuously erupting incisor contains a complete series of developmental stages in a single tooth, i.e., 1) presecretory stage; 2) secretory stage; 3) early maturation stage, and 4) late maturation stage (Warshawsky and Smith, 1974) (Fig. 1).

To date, two major classes of enamel proteins called amelogenins and non-amelogenins have been identified and characterized (Termine *et al.*, 1980a; Ogata *et al.*, 1988; Inage *et al.*, 1989; Deutsch *et al.*, 1991, 1995a). Amelogenins are relatively small (22 to 30 kDa), proline-rich, hydrophobic proteins, which are prone to aggregate. Among the non-amelogenins, enamelins are relatively large (48 to 70 kDa), glycine-rich, hydrophilic proteins, which are acidic and are posttranslationally modified by sugar and phosphate moieties (Limeback and Simic, 1990; Menanteau *et al.*, 1988; Ogata *et al.*, 1988; Fincham *et al.*, 1991). It is broadly accepted that

Abbreviations used in this paper: EDTA, ethylenedinitrilo tetraacetic acid.

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0214-6282/96/$03.00

Printed in Spain

the amelogenins comprise the largest fraction of enamel matrix (Termine *et al.*, 1980b; Deutsch, 1989) in developing teeth and is lost during the maturation stage when the total mineral content of this tissue increases. In addition, several uncharacterized enamel proteins that do not belong to the amelogenin or enamelin protein families have been identified (Graham, 1985; Strawich *et al.*, 1993).

We recently cloned and sequenced ameloblastin, a novel ameloblast-specific protein whose gene maps to mouse chromosome 5 (Krebsbach *et al.*, 1996). Ameloblastin mRNA encodes an open reading frame of 422 amino acids corresponding to a protein of 45 kDa, and it is predicted as an acidic protein (pI= 5.54), rich in proline (15.2%), glycine (9.9%) and leucine (9.9%). From these data, it is likely that ameloblastin does not belong to the amelogenin or enamelin families. In this report, we have studied the expression of ameloblastin in developing rat and human teeth, and compared its expression pattern to amelogenin.

Results

Whole-mount in situ hybridization

Whole-mount preparations of 3-4 week old rat mandibles revealed a linear pattern of expression of ameloblastin mRNA that was restricted to the inner aspect of the enamel epithelium (Fig. 2-1B and Fig. 2-2A, schematic diagram). This expression pattern was similar to that exhibited by amelogenin, but ameloblastin mRNA was distributed more apically and more incisally than the amelogenin mRNA (Fig. 2-1A,B).

Ameloblastin mRNA was also consistently expressed in human tooth germs. Whole-mount *in situ* hybridization revealed that particularly intense expression of ameloblastin mRNA was found in the cuspal regions of early bell stage tooth germs (Fig. 2-1C and Fig. 2-2B, schematic diagram). The secretory ameloblast zone showed strong staining while the immature enamel epithelial zone was negative (Fig. 2-1C). Similar staining was also found in the cervical region of late bell stage tooth germs (Fig. 2-1D), where early stage secretory ameloblasts begin to produce enamel matrix. Although the cuspal enamel epithelium in Figure 2-1D was partly removed during tooth germ extraction, the cervical enamel epithelium which remained closely attached to the forming dentin and enamel matrix expressed ameloblastin mRNA (Fig. 2-1D). *In situ* hybridization in microsection of human bell stage tooth germs showed that cuspal ameloblasts also expressed ameloblastin mRNA (Fig. 2-1E).

In situ hybridization

In situ hybridization sections of paraffin embedded rat incisors showed that ameloblastin expression is limited to the ameloblast cell layer. Presecretory stage ameloblasts first showed expression of ameloblastin mRNA as a diffuse granular pattern throughout their cytoplasm (Fig. 3A-area b, Fig. 3B). Ameloblastin mRNA increased substantially in secretory stage ameloblasts (Fig. 3A-area c, Fig. 3C) and was particularly prominent in early maturation stage ameloblasts (Fig. 3A-area d, Fig. 3D). The expression of ameloblastin persisted at reduced levels in late maturation stage ameloblasts (Fig. 3A-area e, Fig. 3E) where little amelogenin mRNA is expressed (Snead *et al.*, 1988; Lee, unpublished). The *in situ* hybridization signal was characteristically concentrated in the juxtanuclear cytoplasm of secretory, early and late maturation stage ameloblasts. In comparison to the pattern of expression of amelogenin mRNA, ameloblastin mRNA appeared to be expressed earlier during the presecretory stage of development (Fig. 3B).

Immunohistochemistry

Immunolabeling with an antibody to ameloblastin showed intense reaction in ameloblasts and linear staining at the dentino-enamel junction (composite picture of immunostaining in Fig. 4A). No reactivity was seen in the negative control using the antibody preabsorbed with recombinant ameloblastin (Fig. 4J). Ameloblastin was first immunodetected in the distal cytoplasm of preameloblasts (Fig. 4A-area b, Fig. 4B). The labeling was intense in the Tomes' processes of secretory ameloblasts (Fig. 4A-areas c,d,e; Fig. 4C,D,E) and then appeared localized to the distal cytoplasm of early maturation stage ameloblasts. At a later developmental stage, ameloblastin was also weakly detected in the papillary layer of the enamel organ (Fig. 4A-areas g,f, Fig. 4G,F). In late matura-

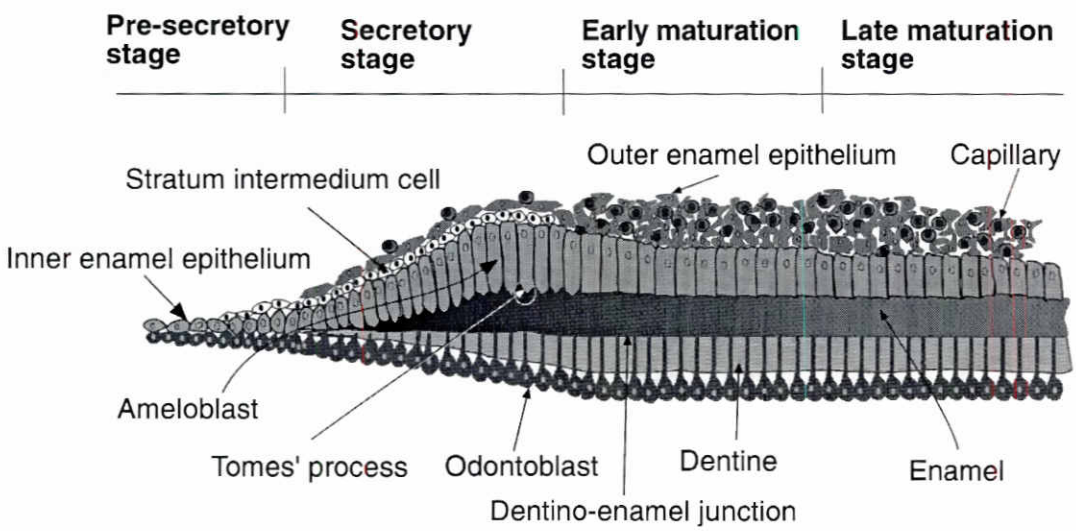


Fig. 1. Schematic representation of the sequential stages of ameloblast cell differentiation.

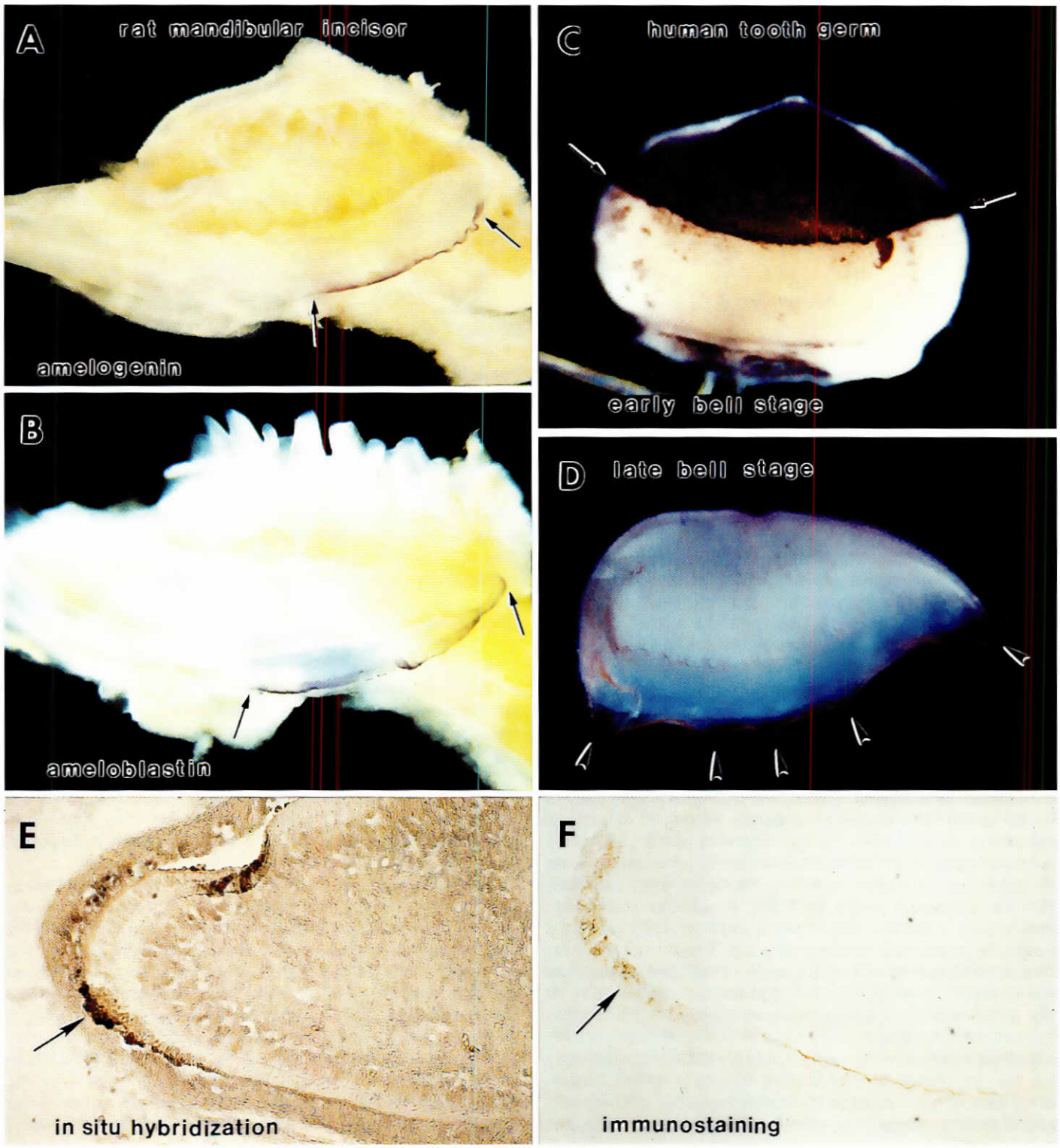


Fig. 2-1. Expression of ameloblastin in rat incisor and human tooth germ. (A,B) *Whole-mount in situ hybridization of 3 week-old rat mandibles.* **(A)** *Amelogenin mRNA expression.* **(B)** *Ameloblastin mRNA expression, ameloblastin mRNA is more widely expressed towards the apical end and the incisal area than amelogenin (extent of expression demarcated by arrows).* **(C,D)** *Whole-mount in situ hybridization of human tooth germs.* **(C)** *Early bell stage; enamel-producing zone in the cuspal area is strongly positive (brown) and shows a clear-cut border (arrows) with the immature ameloblast zone.* **(D)** *Late bell stage; calcification is advanced, and remaining enamel epithelium at the cervical margin (arrows) shows strong reaction (blue).* **(E,F)** *Microsections of human tooth germ (x200);* **(E)** *in situ hybridization of ameloblastin mRNA in microsection of early bell stage of human tooth germs; secretory ameloblasts of the cusp area (arrow) are positive.* **(F)** *Immunostaining with anti-ameloblastin antibody. Positive reaction (brown) is seen in the same ameloblasts (arrow) as seen in E,G*

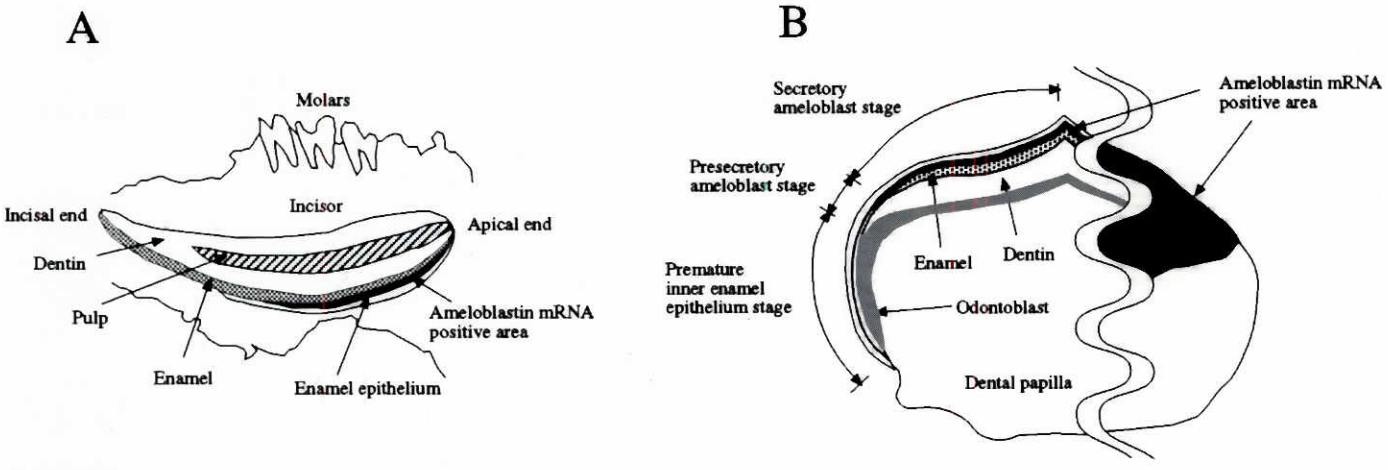


Fig. 2-2. Schematic diagrams of rat incisor and human tooth germ. (A) *Schematic diagram of Fig. 2-1B.* **(B)** *Schematic diagram of Fig. 2-1C. Left half represents a cross-section, and area marked in black indicates the region positive for ameloblastin mRNA expression.*

tion stage ameloblasts, ameloblastin was immunolocalized in the juxtanuclear region (Fig. 4A-areas h,i, Fig. 4H,I). In EDTA-decalcified sections, weak staining could only be detected in the superficial portion (indicated with an arrow) of the enamel matrix (Fig. 4A,D,E,F,G,H). Ameloblastin was also expressed in the ameloblasts of human tooth germs at the bell stage of development (Fig. 2-1F).

High-resolution colloidal gold immunolabeling revealed the presence of ameloblastin antigenicity in the organelles for protein synthesis and secretion (Fig. 5). Saccules of the Golgi apparatus and secretory granules on the trans aspect of this organelle (Fig. 5B) and in Tomes' processes were labeled. Numerous gold particles were also present over the enamel layer (Fig. 5A).

Western blot analysis

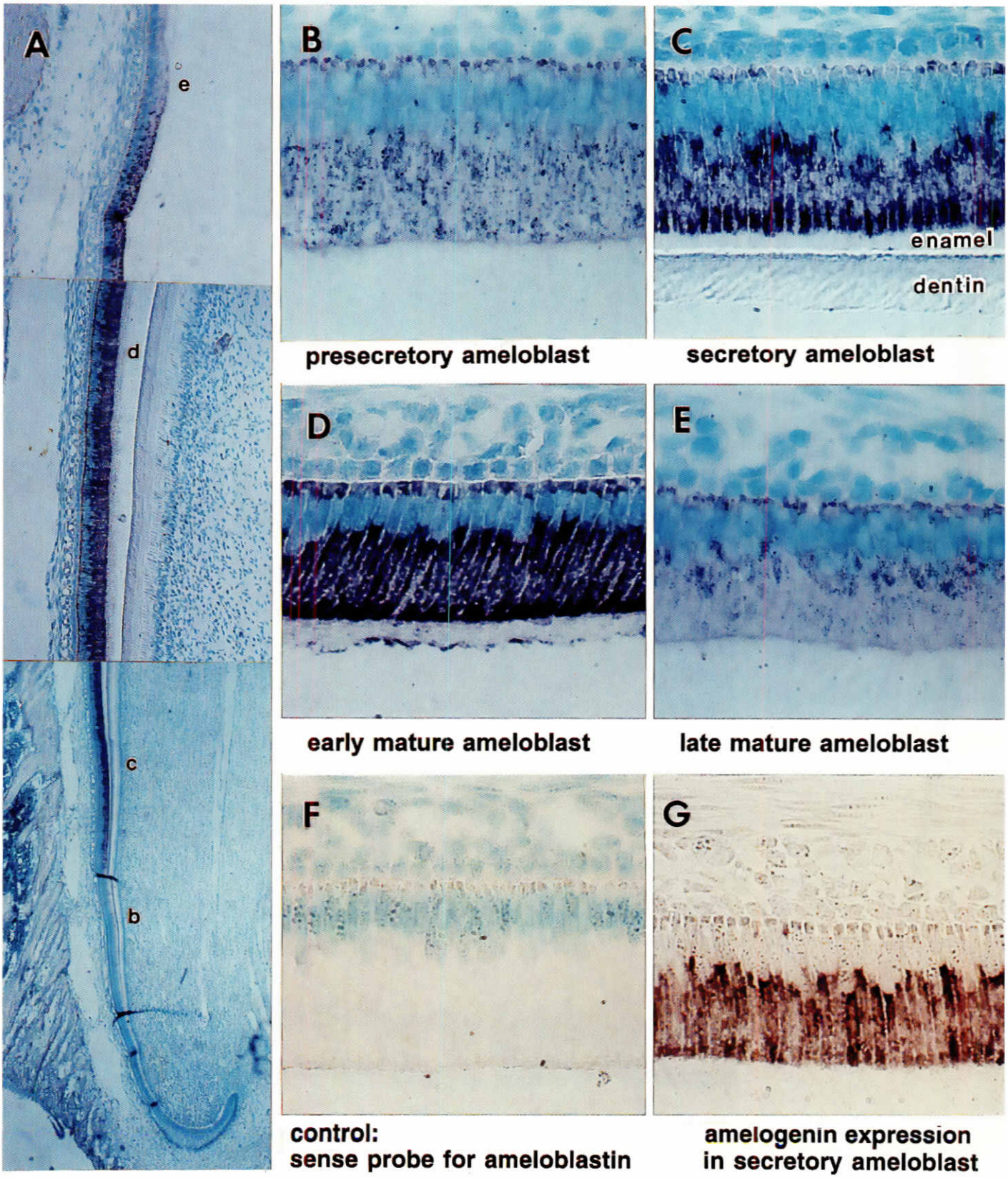
Western blot analysis was performed to identify ameloblastin protein from tooth tissues using an antibody raised against recombinant ameloblastin. The highest molecular weight band of the full length recombinant ameloblastin expressed in bacteria migrated at about 50 kDa (Fig. 6g) consistent with the calculated molecular weight of the recombinant protein together with an extra 20 amino acid sequence containing the histidine-tag sequence from the expression vector (pQE-30). In addition, there were several lower molecular weight bands which probably represent proteolytic degradation products (Fig. 6g). Protein extracts from the enamel epithelial tissue of rat incisors showed several intensely stained protein bands corresponding to approximately 62 kDa, 70 kDa, 84 kDa and 120 kDa in molecular weight (Fig. 6f). The two less intensely staining bands near 47 kDa and 44 kDa correspond to the calculated molecular weight of two forms of precursor ameloblastin that may be derived from alternative splicing of mRNA (unpublished data). Protein extracts of enamel from three different portions of incisors showed major bands of 84 kDa and 120 kDa, and little staining of other smaller molecular weight protein bands (Fig. 6c,d,e). The lower third of incisor roots showed the most intense staining (Fig. 6e) and the upper third the least (Fig. 6c). The EDTA decalcification solution contained 5 moderate to weakly reactive protein bands, the major one situated between 84 and 112 kDa (Fig. 6b). The extract from brain showed no staining with the anti-ameloblastin antibodies (Fig. 6a).

Discussion

Ameloblastin is a unique protein specifically expressed by ameloblasts. The DNA sequence of ameloblastin has no homology with amelogenins and tuftelin, and ameloblastin is mapped to a locus in mouse chromosome 5 (Lau *et al.*, 1989; Deutsch *et al.*, 1991; Bonass *et al.*, 1994; Krebsbach *et al.*, 1996), while amelogenin and tuftelin genes are localized to human X chromosome and chromosome 1, respectively (Bailey *et al.*, 1992; Deutsch *et al.*, 1994). Here, we report the complete developmental expression pattern of ameloblastin in the rat incisor and human tooth germs, and compare its expression to amelogenin.

The secreted enamel matrix is composed of two major classes of proteins, the amelogenins and enamelins (Termine *et al.*, 1980a). The hydrophobic amelogenins comprise over 90% of the secreted extracellular matrix proteins and are rich in proline, histidine and glutamine. The acidic enamelins make up less than 10% of enamel matrix and are rich in glutamic acid, aspartic acid, serine and glycine. Developmental studies suggest that enamel proteins are sequentially expressed, with the anionic enamelins such as tuftelin being synthesized prior to the amelogenins (Zeichner-David *et al.*, 1989). Consequently, acidic enamel proteins would first be deposited along the dentino-enamel junction and appear to function as nucleators (or templates) and regulators of biomineralization (Deutsch, 1989; Slavkin

Fig. 3. *In situ* hybridization in microsections of rat incisor, showing a composite photomicrograph including all developmental stages of the rat incisor. (A,B,C,D,E) *Ameloblastin mRNA expression.* **(A)** *Low power magnification of different stages of ameloblast differentiation (x40). Areas marked b,c,d,e in* **A** *are magnified in panels* **B,C,D,E** *(x400).* **(B)** *Fine granular expression in the cytoplasm of presecretory ameloblasts.* **(C)** *Expression in the juxtanuclear and distal cytoplasm of secretory ameloblasts.* **(D)** *Highest expression is found in early maturation stage ameloblasts.* **(E)** *Some expression remained in the juxtanuclear cytoplasm of late maturation stage ameloblasts.* **(F)** *Negative control; RNA in situ hybridization using the sense probe of ameloblastin shows no reaction (x400).* **(G)** *RNA in situ hybridization of amelogenin; note the condensed expression in the distal cytoplasm of secretory ameloblasts (x400).*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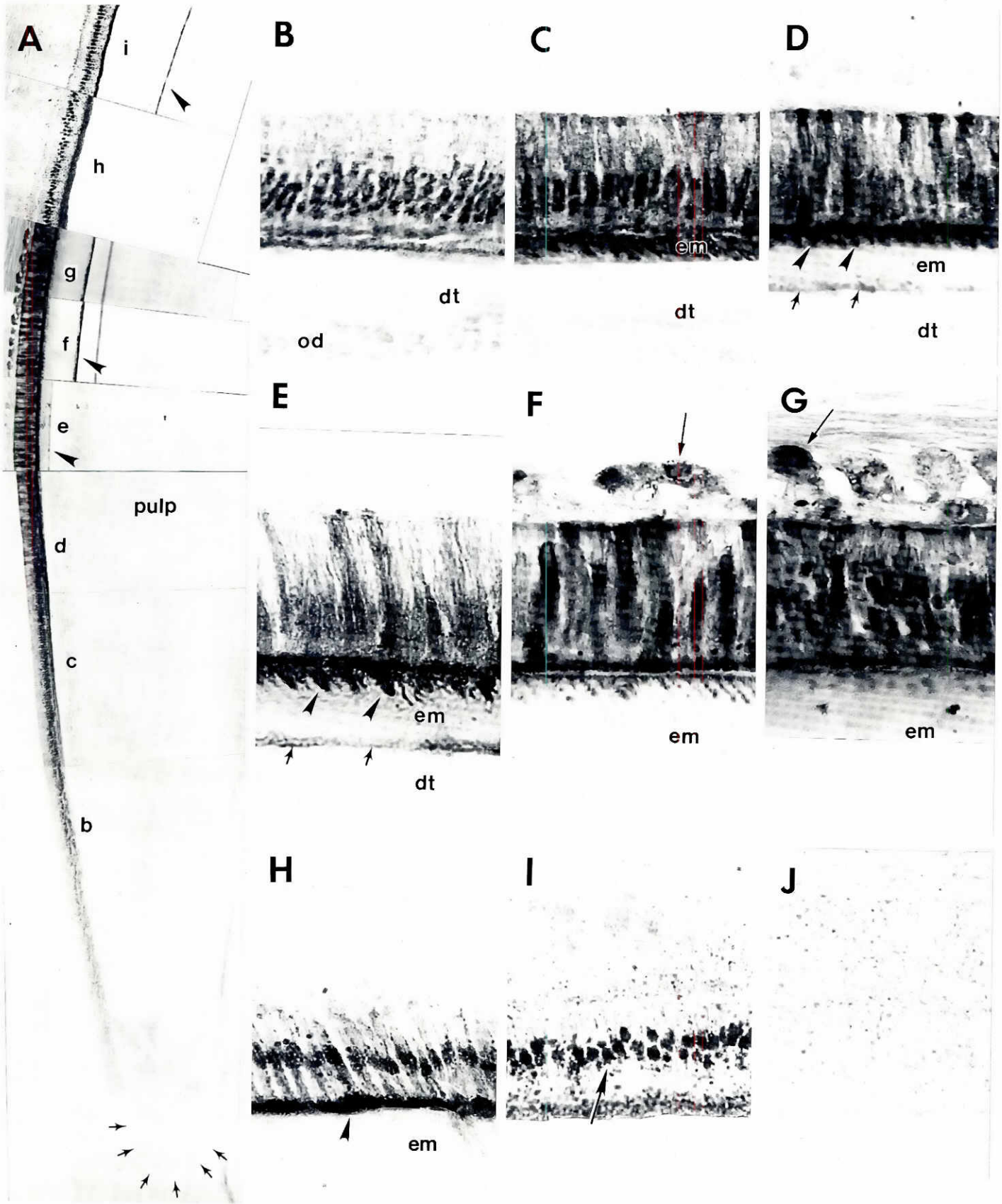
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et al., 1992). The more hydrophobic amelogenins are synthesized approximately 48 h after the anionic murine tuftelin and appear to function as calcium chelating nucleating sites (Glimcher, 1989),inhibitors of crystal growth (Doi *et al.*, 1984; Aoba *et al.*, 1987) and/or regulators of crystal size, growth and orientation (Fearnhead, 1979; Aoba *et al.*, 1989; Robinson *et al.*, 1989; Fincham *et al.*, 1991; Deutsch *et al.*, 1995b).

We used the continuously erupting rat incisor as a model system to determine the expression pattern of ameloblastin, because the entire developmental sequence of amelogenesis can be studied in a single tooth. *In situ* hybridization, immunostaining, and Western blotting techniques were applied to compare ameloblastin expression to that of amelogenin, the major secretory product of ameloblasts. By *in situ* hybridization, ameloblastin mRNA was first observed in presecretory ameloblasts, at which stage the Golgi apparatus and rough endoplasmic reticulum are already well developed (Smith and Nanci, 1989b). Ameloblastin protein gradually accumulated in the distal cytoplasm of ameloblasts, particularly in the Tomes' processes of secretory stage ameloblasts. Diffuse immunostaining was found in the superficial enamel matrix and the dentino-enamel junction. Colloidal gold immunolabeling further showed that ameloblastin is present in the Golgi apparatus, secretory granules, and throughout enamel. These immunocytochemical observations indicate that ameloblastin is a secretory product of ameloblasts.

Ameloblastin was also highly expressed in ameloblasts of human tooth germs. Ameloblastin protein was deposited in the enamel matrix of early bell stage tooth germs, and ameloblastin mRNA showed a stage-specific pattern of expression that was abundant in secretory stage ameloblasts. These data suggest that ameloblastin is a phylogenetically conserved protein which plays a fundamental role in enamel formation.

The neutral EDTA decalcified incisor sections showed little immunostaining for ameloblastin in the enamel matrix. However, the frozen sections showed a strong positive reaction in the enamel matrix, and the neutral EDTA decalcification solution itself showed a weak positive reaction in Western blot analysis. This suggests that ameloblastin is extracted by neutral EDTA during the decalcification procedure, which is a chemical property that contrasts with the known solubility property of amelogenin (Fincham *et al.*, 1991).

In the continuously erupting rat incisor, early maturation stage ameloblasts begin to show immunolocalization of ameloblastin into the enamel epithelium, and late maturation stage ameloblasts showed an irregular accumulation of ameloblastin in the juxtanuclear cytoplasm as well as in the distal cytoplasm near the enamel surface. Weak immunostaining was continuous in the papillary layer of enamel epithelium until the late maturation stage. However, the cytoplasmic accumulation as large granular bodies was not seen in the immunostaining patterns of amelogenin and enamelin (Slavkin *et al.*, 1982; Snead *et al.*, 1988; Zeichner-David *et al.*, 1995). Thus, these findings suggest that there may be multiple roles of ameloblastin for enamel maturation in the late maturation stage ameloblasts, or that ameloblastin could be degraded and ingested by mature ameloblasts, resulting in cytoplasmic vesicular accumulations. Discrete and progressive protein degradation by specific proteases have been implicated in protein removal from developing teeth (Shimizu *et al.*, 1979; Nanci *et al.*, 1987; Carter *et al.*, 1989; Smith *et al.*, 1989), but the detailed mechanism of such processes needs to be elucidated.

Western blot analysis showed that both enamel epithelial tissue and enamel matrix contained several protein bands strongly stained with the antibodies to ameloblastin whose sizes are larger than the molecular weight calculated from the deduced protein sequence. These proteins probably represent a posttranslational modification(s) of precursor ameloblastin. Such modifications could include phosphorylation, glycosylation and sulfation. Alternatively, these proteins may represent a protein complex with other enamel proteins. In the enamel epithelial tissue, there are two proteins, 47 kDa and 44 kDa, which are less abundant than the proteins with higher molecular weight. Since there are at least two variant mRNAs for ameloblastin differed by 45 bp in size which are likely the result of alternative splicing, these proteins may correspond to unmodified precursor ameloblastin translated from the two different size mRNAs. Interestingly, the enamel epithelial tissue contains two strongly expressed proteins, 62 kDa and 70 kDa, which are not found in the enamel matrix. It is possible that these proteins may originate from a transcript which does not have a signal peptide and which may function intracellularly in a certain stage, especially the late maturation stage of amelogenesis in which ameloblastin is still expressed whereas amelogenin and tuftelin are not. Ameloblastin may have broader functions than amelogenin since its expression continues to the late stage of ameloblast differentiation. Thus, it will be important to examine if ameloblastin has multiple functions throughout amelogenesis.

Materials and Methods

RNA probes for in situ hybridization

The 2.0 kb full-length ameloblastin cDNA clone Y224 (Matsuki *et al.*, 1995) was linearized by BamHI and XhoI for antisense and sense probe production, respectively. The 0.8 kb amelogenin cDNA clone Y166 (Matsuki *et al.*, 1995) was linearized by BamHI and HindIII for antisense and sense probe production, respectively. Digoxigenin-UTP labeled, single-stranded antisense and sense RNA probes were prepared with T7 RNA polymerase and T3 RNA polymerase, respectively, using an RNA labeling kit (Boehringer Mannheim, Indianapolis, IN, USA).

Ameloblastin antibody preparation

A recombinant protein was generated by subcloning a fragment of Y224 corresponding to +613 to +1134 bp into the expression vector pQE-30,

Fig. 4. Immunostaining of ameloblastin in microsections of rat incisor. (A-I) *No counter-staining.* **(A)** *Low power composite reconstruction showing adjacent stages of ameloblast differentiation; note the positive intracellular immunoperoxidase reactivity up to the apical end of the incisor, but not including the apical end (small arrows), and positive reaction at the dentino-enamel junction (arrowheads) (x40). The regions labeled b,c,d,e,f,g,h,i in* **A** *are shown magnified in* **B,C,D,E,F,G,H,** *and* **I**. **(B)** *Presecretory stage (x400); ameloblastin appears in the distal cytoplasm of ameloblasts, but is negative in dentin (dt) and odontoblasts (od).* **(C,D,E)** *Secretory stages (x400). In* **C**, *the enamel (em) shows condensed staining for ameloblastin. In* **D**, *the dentinoenamel junction (arrows) shows positive reaction and Tomes' processes (arrowheads) develop strong staining, but the enamel becomes negative. In* **E**, *staining of Tomes' processes remains generally strong.* **(F,G)** *Early maturation stage (x400).* **(F)** *Tomes' processes are generally retracted or flattened, and ameloblastin is positive in the superficial layer of enamel. Note also the positivity in the outer enamel epithelium (arrow).* **(G)** *Ameloblastin becomes concentrated in the cytoplasm as irregular, large granules, and there is increased staining in the outer enamel epithelium (arrow).* **(H,I)** *Late maturation stage x400).* **(H)** *Ameloblastin remains condensed on the surface layer of enamel (arrowhead).* **(I)** *Ameloblastin gradually accumulates in the proximal cytoplasm (arrow).* **(J)** *Negative control after preabsorption of the antibody by the immunizing fusion protein shows no staining (x400).*

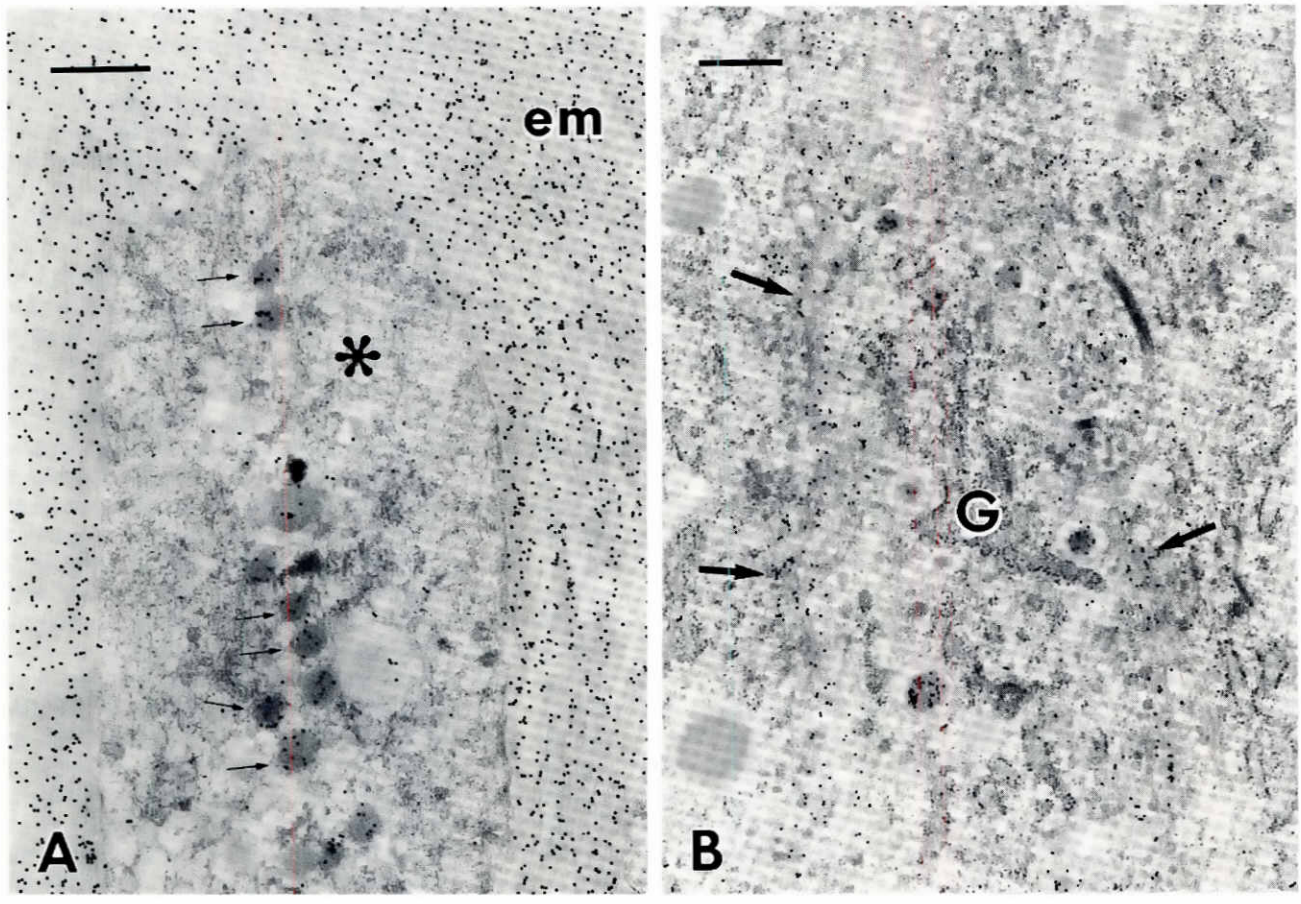

Fig. 5. Immunogold preparations showing the presence of ameloblastin antigenicity in the protein synthetic and secretory organelles of secretory stage ameloblastin. (A) *Secretory granules (arrows) accumulating in Tomes' process (*) and the surrounding enamel (em) show numerous gold particles (bar, x26,000).* **(B)** *The saccules (arrows) of the Golgi apparatus (G) as well as secretory granules on their trans aspect are also intensely immunolabeled. Gold particles are also occasionally seen in association with endoplasmic reticulum proper (arrowhead). Bar, x21,000.*

which contains a tag consisting of six histidine codons (Qiagen, Chatsworth, CA, USA). The plasmid was transformed into competent M15(pREP4) cells, and the recombinant protein was induced with isopropyl-ß-D-thiogalactopyranoside and purified using a Ni-nitrilo-tri-acetic-acid agarose affinity column (Qiagen, Chatsworth, CA, USA). Polyclonal antibodies against the purified recombinant ameloblastin were produced using standard procedures. New Zealand white rabbits were inoculated with 500 μg of recombinant ameloblastin in an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA). The rabbit was boosted 3 times at 2 week intervals using 250 μg of recombinant ameloblastin and an equal volume of Freund's incomplete adjuvant, and polyclonal ameloblastin antibody was generated (Duncroft Inc, VA, USA). Isolation of IgG from serum was performed by recombinant-protein antigen-affinity purification using the recombinant protein bound to a Ni-nitrilo-tri-acetic acid agarose column.

Whole-mount in situ hybridization

Three-week old Sprague-Dawley rats were anesthetized with ketamine hydrochloride (Sigma, St. Louis, MO, USA), and were perfused through the left ventricle with 4% paraformaldehyde in PBS (pH 7.0). The mandibles were excised, decalcified in 10% EDTA (pH 8.0) for 10 days, and bisected longitudinally to expose the incisor. Fixed sections of human fetal tooth germs were kindly provided by the Registry of Congenital Malformation administered by the Department of Pathology, Seoul National University College of Medicine. They were diagnosed as non-pathologic in routine pathological examinations. The human fetal tooth germs at 20, 24 and 30 weeks of gestation were extracted during autopsy. Tooth germs were fixed in 10% neutral formalin and maintained in 70% ethanol solution at -70°C until analyzed. Whole-mount RNA *in situ* hybridization was performed using single-stranded sense and antisense probes (see above) using digoxigenin-labeled, *in vitro* transcribed RNA probes (Wilkinson, 1992).

RNA in situ hybridization

Three week-old Sprague-Dawley rats and human tooth germs were fixed as described above for whole-mount *in situ* hybridization. Specimens were embedded in paraffin and 5 μm sections were prepared. After deparaffinization, the sections were treated with proteinase K (10 μg/ml) for 15 min at room temperature, and endogenous alkaline phosphatase was inactivated using 0.2 N HCl. Hybridization in the tissue sections was performed in a hybridization solution containing 50% formamide, 10 mM Tris-HCl, pH 7.6, 200 μg/ml tRNA, 1x Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA at 50°C for 16 h in a humidified chamber. Slides were washed in 2xSSC containing 50% formamide at 55°C for 30 min, and then rinsed at 37°C for 10 min in TNE (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA). Non-hybridized transcripts were digested with 20 μg/ml RNase A (Sigma, St. Louis, MO, USA) in TNE at 37°C for 30 min. The slides were washed again in TNE at 37°C for 10 min, once with 2xSSC at 50°C for 20 min, and finally twice with 0.2xSSC at 50°C for 20 min.

Detection of *in situ* hybridization was carried out using the Genius Detection system (Boehringer Mannheim, Indianapolis, IN, USA), in which

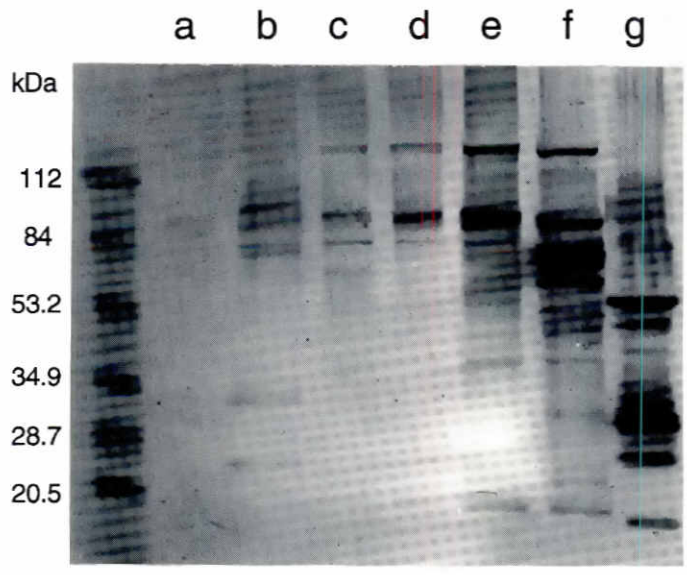

Fig. 7. Western blot analysis for ameloblastin; ameloblastin is seen in EDTA-decalcification solution as well as in various rat incisor tissues. *Rat incisors were treated with EDTA and the soluble fraction was analyzed. In addition intact calcified incisors without EDTA treatment were dissected into apical, cervical, and incisal portions and were homogenized by Polytron in sample buffer. The soluble fractions from these tissues were fractionated by SDS 4-20% polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred into a Nylon filter followed by staining with anti-recombinant ameloblastin antibody.* **(a)** *Control, rat brain tissue.* **(b)** *EDTA decalcification solution of rat incisors.* **(d)** *Cervical third of rat incisor.* **(e)** *Apical third of rat incisor.* **(f)** *Enamel epithelial tissue.* **(g)** *Recombinant ameloblastin using full-length cDNA.*

the specific transcripts were detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase in DIG 1 solution. The slides were washed several times with DIG 3 solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and then immersed in the color-development solution (0.3 mg/ml Nitro Blue Tetrazolium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M $NaHCO_3$) (Boehringer Mannheim, Indianapolis, IN, USA). Color development was stopped by immersing the slides in DIG 4 solution (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA).

Immunohistochemistry

Paraffin sections of rat incisors and human tooth germs were deparaffinized and hydrated before inactivation of endogenous peroxidases by 3% hydrogen peroxide for 10 min. The sections were blocked with normal goat serum, and three-layer immunolabeling was performed. For immunolabeling, an antigen-affinity purified antibody raised against recombinant ameloblastin was used. The sections were incubated with a biotinylated secondary anti-rabbit IgG antibody followed by streptavidin-peroxidase. Immunolocalization was visualized by diaminobenzidine (HistoMark, KPL, Gaithersburg, MD, USA). As a negative control, the antibody against ameloblastin was preabsorbed with the recombinant protein and then used in the same immunostaining procedure described above.

For postembedding colloidal gold immunolabeling, male Wistar rats (Charles Rivers, St-Constant, QC) weighing approximately 100 g were anesthetized with chloral hydrate (0.4 mg/g body weight) and perfused through the left ventricle into the aorta with lactated Ringer's solution (Abbott Laboratories, Montreal, Canada) for approximately 30 sec followed by 1% glutaraldehyde buffered with 0.08 M sodium cacodylate containing 0.5% $CaCl_2$ (pH 7.2-7.4). Hemimandibles were dissected out and immersed overnight in the perfusion fixative. Segments of incisors containing early secretory stage ameloblasts were prepared using a molar reference line (Smith and Nanci, 1989a). The segments were washed extensively in 0.1 M sodium cacodylate buffer containing 0.5% $CaCl_2$ and 5% sucrose (pH 7.2-7.4), dehydrated in graded methanols and processed in Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, Germany) as described previously (Bendayan *et al.*, 1987; Nanci *et al.*, 1989). Thin sections were cut, mounted on formvar-carbon coated nickel grids and processed for colloidal gold immunolabeling using the rabbit antiserum to recombinant ameloblastin (see above). The sections were then stained with uranyl acetate and lead citrate and examined in a JEOL 1200EX-II electron microscope operated at 60 kV.

Western blot analysis

Incisor roots were carefully removed from 3 week-old Sprague-Dawley rats, and the enamel epithelial tissue was excised from the labial surfaces of incisors under a dissecting microscope. The tissue was homogenized in sample buffer (0.6 M Tris-HCl, pH 6.8, 2% SDS) using a Polytron (Tekmar, Cincinnati OH, USA). The remaining incisor root tissues were washed in 6% H_2O_2 PBT solution (phosphate buffered saline, 0.1% Tween-20) for 2 h on a rocking platform at room temperature. Any remaining soft tissue was gently removed, and the incisor roots with the exposed enamel surface were then decalcified in a 10% EDTA solution (pH 7.5) for 10 days. The decalcification solution was collected, lyophylized, and resuspended in sample buffer. Non-decalcified incisors, without EDTA treatment, were evenly cut into three pieces: the upper third, the middle third, and the lower third. Each piece of the incisors was homogenized in sample buffer, and was centrifuged for 20 min at 3000 rpm (Beckman, RJ6), and the supernatants were used for Western blot analysis. Recombinant ameloblastin was also made by subcloning a fragment of Y224 encoding the entire ameloblastin open reading frame (nucleotide residues from +92 to +1359) into pQE-30. The pQE-30 vector was designed to express an additional 20 amino acid sequence including a six-histidine tag for column purification. Rat brain extract was used as a negative control. The samples were treated with protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), aprotonin (0.3 mM), and leupeptin (1 mM) (Boehringer Mannheim, Indianapolis, IN, USA) during the extraction procedure. The protein extracts were fractionated by electrophoresis through a 4-20% polyacrylamide gel (Novex, San Diego, CA, USA) in the presence of sodium dodecyl sulfate buffer under reducing conditions. The electrophoresed proteins were then transferred to a Nylon filter (PVDF, Millipore, Bedford, MA, USA). Immediately after transfer, the filter was blocked in TBST buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% dry non-fat-milk for 30 min at room temperature, was washed in TBST and transferred to a fresh solution of TBST containing a 1:1000 dilution of the antibody, and incubated at room temperature overnight. The filter was washed with TBST and then incubated with a 1:2000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase for 2 h. After washing several times with TBST, the filter was immersed in the color-development solution (0.3 mg/ml Nitro Blue Tetrazolium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M $NaHCO_3$) (Boehringer Mannheim, Indianapolis, IN, USA). The color reaction was stopped by immersing the filter in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Acknowledgments

This paper was supported in part by a grant from the MRC of Canada to A.N, and we thank Dr. J. Hashimoto for helping with the colloidal gold preparation.

References

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-termini of amelogenin in protein-enamel mineral interaction. *J. Dent. Res. 68:* 1331-1336.

BAILEY, D.M., AFFARA, N.A., FERGUSON-SMITH, M.A., TANABE, T., FUKAE, M., UCHIDA, T. and SHIMIZU, M. (1992). The X-Y homologous gene amelogenin maps to the short arms of both the X and Y chromosomes and is highly conserved in primates. The localization and characterization of proteinases for the initial cleavage of porcine amelogenin. *Genomics 14:* 203-205.

BENDAYAN, M., NANCI, A. and KAN, F.W. (1987). Effect of tissue processing on colloidal gold cytochemistry. *J. Histochem. Cytochem. 35:* 983-996.

BONASS, W.A., ROBINSON, P.A., KIRKHAM, J., SHORE, R.C. and ROBINSON, C. (1994). Molecular cloning and DNA sequence of rat amelogenin and a comparative analysis of mammalian amelogenin protein sequence divergence. *Biochem. Biophys. Res. Commun. 198:* 755-763.

CARTER, J., SMILLIE, A.C. and SHEPHERD, M.G. (1989). Purification and properties of a protease from developing porcine dental enamel. *Arch. Oral. Biol. 34:* 195-202.

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

DEUTSCH, D., CATALANO-SHERMAN, J., DAFNI, L., DAVID, S. and PALMON, A. (1995a). Enamel matrix proteins and ameloblast biology. *Connect. Tissue. Res. 32:* 97-107.

DEUTSCH, D., PALMON, A., DAFNI, L., CATALANO-SHERMAN, J., YOUNG, M.F. and FISHER, L.W. (1995b). The enamelin (tuftelin) gene. *Int. J. Dev. Biol. 39:* 135-143.

DEUTSCH, D., PALMON, A., FISHER, L.W., KOLODNY, N., 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., PALMON, A., YOUNG, M.F., SELIG, S., KEARNS, W.G., FISHER, L.W. and WANG, W. (1994). Mapping of the human tuftelin (TUFT1) gene to chromosome 1 by fluorescence *in situ* hybridization. [Ectopic bone induction by human fetal enamel proteins]. *Mammal. Genome 5:* 461-462.

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.

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

FINCHAM, A.G., HU, Y., LAU, E.C., SLAVKIN, H.C. and SNEAD, M.L. (1991). Amelogenin post-secretory processing during biomineralization in the postnatal mouse molar tooth. *Arch. Oral Biol. 36:* 305-317.

GLIMCHER, M. (1989). Mechanism of calcification: role of collagen fibrils and collagen-phosphoprotein complexes *in vitro* and *in vivo*. *Anat. Rec. 224:* 139-153.

GRAHAM, E.E. (1985). Isolation of enamel-like proteins from blue shark *(Prionace glauca)* enameloid. *J. Exp. Zool. 234:* 185-191.

INAGE, T., SHIMOKAWA, H., TERANISHI, Y., IWASE, T., TODA, Y. and MORO, I. (1989). Immunocytochemical demonstration of amelogenins and enamelins secreted by ameloblasts during the secretory and maturation stages. *Arch. Histol. Cytol. 52:* 213-29.

KOLLAR, E.J. and MINA, M. (1991). Role of the early epithelium in the patterning of the teeth and Meckel's cartilage. *J. Craniofac. Genet. Dev. Biol. 11:* 223-228.

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

LAU, E.C., MOHANDAS, T.K., SHAPIRO, L.J., SLAVKIN, H.C. and SNEAD, M.L. (1989). Human and mouse amelogenin gene loci are on the sex chromosomes. *Genomics 4:* 162-8.

LIMEBACK, H. and SIMIC, A. (1990). Biochemical characterization of stable high molecular-weight aggregates of amelogenins formed during porcine enamel development. *Arch. Oral Biol. 35:* 459-468.

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:* 55-169.

MATSUKI, Y., NAKASHIMA, M., AMIZUKA, N., WARSHAWSKY, H., GOLTZMAN, D., YAMADA, K.M. and YAMADA, Y. (1995). A compilation of partial sequences of randomly selected cDNA clones from the rat incisor. *J. Dent. Res. 74:* 307-312.

MENANTEAU, J., MEFLAH, K. and STRECKER, G. (1988). The carbohydrate moiety of mineral-bound proteins from fetal enamel: a basis for enamelins heterogeneity. *Calcif. Tissue Int. 42:* 196-200.

NANCI, A., AHLUWALIA, J.P., POMPURA, J.R. and SMITH, C.E. (1989). Biosynthesis and secretion of enamel proteins in the rat incisor. *Anat. Rec. 224:* 277-291.

NANCI, A., SLAVKIN, H.C. and SMITH, C.E. (1987). Immunocytochemical and radioautographic evidence for secretion and intracellular degradation of enamel proteins by ameloblasts during the maturation stage of amelogenesis in rat incisors. *Anat. Rec. 217:* 107-123.

OGATA, Y., SHIMOKAWA, H. and SASAKI, S. (1988). Purification, characterization, and biosynthesis of bovine enamelins. *Calcif. Tissue. Int. 43:* 389-399.

ROBINSON, C., KIRKHAM, J. and FINCHAM, A. (1989). The enamelin/non-amelogenin problem. A brief review. *Connect. Tissue Res. 22:* 93-100.

RUCH, J. V., LESOT, H. and BEGUE-KIRN, C. (1995). Odontoblast differentiation. *Int. J. Dev. Biol. 39:* 51-68.

SHIMIZU, M., TANABE, T. and FUKAE, M. (1979). Proteolytic enzyme in porcine immature enamel. *J. Dent. Res. 58B:* 782-788.

SLAVKIN, H.C., BESSEM, C., BRINGAS, P. Jr., ZEICHNER-DAVID, M., NANCI, A. and SNEAD, M.L. (1988). 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., HU, C.C., SAKAKURA, Y., DIEKWISCH, T., CHAI, Y., MAYO, M., BRINGAS, P. Jr., SIMMER, J., MAK, G., SASANO, Y. *et al.* (1992). Gene expression, signal transduction and tissue-specific biomineralization during mammalian tooth development. *Crit. Rev. Eukaryot. Gene Expr. 2:* 315-329.

SLAVKIN, H., ZEICHNER-DAVID, M., MACDOUGALL, M., BRINGAS, P., BESSEM, C. and HONIG, L.S. (1982). Antibodies to murine amelogenins: localization of enamel proteins during tooth organ development *in vitro*. *Differentiation 23:* 73-82.

SMITH, C.E. and NANCI, A. (1989a). A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat. Rec. 225:* 257-266.

SMITH, C.E. and NANCI, A. (1989b). Secretory activity as a function of the development and maturation of ameloblasts. *Connect. Tissue Res. 22:* 147-156.

SMITH, C.E., POMPURA, J.R., BORENSTEIN, S., FAZEL, A. and NANCI, A. (1989). Degradation and loss of matrix proteins from developing enamel. *Anat. Rec. 224:* 292-316.

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., 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.M. and NYLEN, M. U. (1980a). Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. *J. Biol. Chem. 255:* 9760-9768.

TERMINE, J.D., BELCOURT, A.B., MIYAMOTO, M.S., and CONN, K.M. (1980b). Properties of dissociatively extracted fetal tooth matrix proteins. II. Separation and purification of fetal bovine dentin phosphoprotein. *J. Biol. Chem. 255:* 9769-9772.

WARSHAWSKY, H., and SMITH, C.E. (1974). Morphological classification of rat incisor ameloblasts. *Anat. Rec. 179:* 423-446.

WILKINSON, D.G. (1992). *In Situ Hybridization: A Practical Approach.* Oxford University Press, New York.

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 differentiation. *Int. J. Dev. Biol. 39:* 69-92.

ZEICHNER-DAVID, M., MacDOUGALL, M., DAVIS, A., VIDES, J., SNEAD, M. and SLAVKIN, H. (1989). Enamelins and amelogenins share the same amino-terminal sequence. *Connect Tissue. Res. 22:* 123-129.

Accepted for publication: September 1996